IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:
UMAÑA *et al.*.
Appl. No.: 10/633,697
Filed: August 5, 2003
For: **Glycosylation Engineering of Antibodies for Improving Antibody-Dependent Cellular Cytotoxicity**

Confirmation No.: 5455
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Examiner: Guzo, D.
Atty. Docket: 1975.0010005/TJS

Declaration Under 37 C.F.R. § 1.132 of Pablo Umaña, Ph.D.

Commissioner for Patents
PO Box 1450
Alexandria, VA 22313-1450

Sir:

I, Pablo Umaña, Ph.D., declare and state as follows:

1. I am an inventor of the above-captioned application, U.S. Patent Application Number 10/633,697, filed August 5, 2003, entitled "Glycosylation Engineering of Antibodies for Improving Antibody-Dependent Cellular Cytotoxicity."

2. I am currently Chief Scientific Officer and Member of the Board at GlycArt Biotechnology AG in Zurich, Switzerland. I am an expert in the fields of molecular biology and immunology, with particular expertise in the area of antibody engineering. My qualifications as an expert are established by the attached *curriculum vitae* and the publications listed therein. (Exhibit A)

3. I have read and understood the subject matter of U.S. Pat. Appl. No. 10/633,697.

4. Glycoengineered antibodies produced according to the techniques we described in the above-captioned patent application are characterized by a substantial increase in the proportion of nonfucosylated oligosaccharides in their Fc region and

exhibit significantly increased binding to Fc receptors and antibody-dependent cellular cytotoxicity. Our studies confirming this are set forth in the attached Exhibits B-D.

5. The present application teaches methods for producing recombinant antibodies with increased Fc-mediated cellular cytotoxicity and/or increased Fc receptor binding by engineering a host cell to express an antibody and to have an altered level of activity of at least one glycoprotein-modifying glycosyltransferase. According to one embodiment, the application teaches that the altered level of glycosyltransferase activity is achieved by expression of at least one glycoprotein-modifying glycosyltransferase. The application specifically identifies GnT III and ManII as useful glycoprotein modifying glycosyltransferases for this purpose. In addition, the application teaches that glycoengineered antibodies can be obtained by coexpressing the antibody with *multiple* glycoprotein-modifying glycosyltransferases, such as GnTIII + ManII. (*See* page 13, lines 18-30; page 21, lines 15-25.)

6. Exhibit B is U.S. Pat. Appl. Pub. No. U.S. 2004/0241817, which published December 2, 2004, and is entitled "Fusion Constructs and Use of Same to Produce Antibodies with Increased Fc Receptor Binding Affinity and Effector Function." I am a named inventor on that application. Example 6 of the application describes experiments that I and my colleagues conducted to study the effect of coexpression in a host cell of the human α-Mannosidase II ("hManII") gene or, alternatively, a polynucleotide encoding the Golgi localization domain of hManII fused to the catalytic domain of human galactosyltransferase, and an expression vector encoding the recombinant anti-CD20 antibody C2B8.

7. Specifically, to study the effect of hManII coexpression, we transfected HEK 293-EBNA cells with expression vectors encoding each of the light chain and

heavy chain of the anti-CD20 monoclonal antibody and the hManII gene. At day 5 post-transfection, supernatant was harvested and the monoclonal antibody was purified from the supernatant by sequential chromatography. (Paragraph Nos. 0434 - 0436.)

8. We analyzed the oligosaccharide structures of the purified antibody by enzymatically cleaving the oligosaccharides from the antibodies by PNGaseF digestion and, optionally, EndoH glycosidase digestion. The enzymatic digests containing the released oligosaccharides were subsequently analyzed by MALDI/TOF-MS. (Paragraph Nos. 0437-0446.)

9. The oligosaccharide profile of the anti-CD20 antibody coexpressed with hManII and the relative percentages of the structures found associated to the Fc portion of the antibody are shown in FIG. 34. As noted in the application, the oligosaccharides found associated to the Fc portion of the antibody are complex structures, 48% of which lack core fucose. The α-Mannosidase II competes with the core-fucosyltransferase, generating 48% non-fucosylated oligosaccharide structures. In the absence of α-Mannosidase II, the oligosaccharides of the Fc portion of the antibody are composed of only fucosylated structures (wild-type antibody). (*See* Paragraph No. 0448).

10. The oligosaccharide profile of the anti-CD20 antibody coexpressed with the hManII-GalT fusion protein is shown in FIG. 35A-B. Antibodies produced in the presence of hManII-GalT show almost 100% non-fucosylated structures. (Paragraph No. 0449.)

11. We also assayed antibodies produced in the presence of hManII or hManII-GalT fusion protein for binding to Fc receptors and ADCC activity. As shown in FIG 29, expression of a ManII-GnTIII fusion polypeptide in the antibody-producing host cells resulted in an increase in ADCC activity. (Paragraph No. 0419.) The binding

assay experiment utilized the cell line CHO-1708-37, which expresses on its surface the FcγRIIIA receptor (CD16) and the γ chain of the FcγRI receptor. The expression of the FcγRIIIA receptor was assessed by FACS analysis using the 3G8-FITC monoclonal antibody. As shown in FIG. 36, the anti-CD20 antibody produced in the presence of α-Mannosidase II binds to the FcγRIIIA receptor with higher affinity than the wild-type antibody that was not produced in the presence of ManII. (Paragraph No. 0457.)

12. Exhibit C is a paper by my colleagues and me that was published in the journal, *Biotechnology & Bioengineering* 93:851-61 (2006). The title of the paper is "Modulation of Therapeutic Antibody Effector Functions by Glycosylation Engineering." In general, it describes the generation of anti-CD20 antibody glycovariants by coexpression in a host cell of GnT-III or chimeric GnTIII proteins.

13. In these studies, we analyzed two anti-CD20 antibodies produced by either: 1) coexpression with a fusion protein comprising the catalytic domain of human GnTIII fused to the Golgi localization domain of human Mannosidase II (termed "Glyco-1" antibodies); or 2) coexpression with the GnT-III/ManII fusion construct and a gene encoding ManII ("Glyco-2" antibodies) for ADCC activity and for their affinity to bind FcγRIIIa compared to either the unmodified (*i.e.*, not glycoengineered) antibody, or to an antibody glycovariant produced under the same conditions by co-expression of GnT-III ("Glyco-0"). (Pages 856-857.)

14. We evaluated ADCC of the glycoengineered antibodies and found that both the Glyco-1 and Glyco-2 antibodies mediate an enhanced ADCC response. (Page 857; FIG. 5A.) The Glyco-1 and Glyco-2 variants were also 100-fold more potent than unmodified antibodies in depleting B-cells. (Page 857; FIG 5C.)

15. We also evaluated the binding of the glycoengineered antibodies to FcγRIIIA on peripheral human natural killer (NK) cells, which are known to be important mediators of ADCC, and to constitutively express FcγRIIIA. Both Glyco-1 and Glyco-2 were found to bind with a considerably higher affinity to NK cells than unmodified antibody. (*See* FIG. 4A). The Glyco-0 antibody produced by wild-type GnT-III coexpression was found to have intermediate FcγRIIIA binding affinity between that of the unmodified antibody and Glyco-1/Glyco-2. (Page 857.)

16. Exhibit D shows the results of a study we conducted to determine the effect of constitutive coexpression of recombinant, wild-type β1,4-N-acetylglucosaminyltransferase III (GnT-III) *and* Golgi α-mannosidase II (ManII) in stable, industrial grade CHO cells on a recombinant anti-CD20 antibody produced by those cells.

17. FIG. 1 of Exhibit D shows the glycosylation profile (as determined by MALDI/TOF-MS) for the Fc-region oligosaccharides of a nonglycoengineered recombinant antibody produced in CHO cells. The level of nonfucosylated oligosaccharides is below 10%, which is typical for a nonglycoengineered antibody produced by CHO cells.

18. FIG 2 of Exhibit D shows the glycosylation profile for the Fc-region oligosaccharides of a *glycoengineered* anti-CD20 antibody produced in CHO cells. The glycoengineered antibodies had significantly increased levels of nonfucosylated oligosaccharides relative to the nonglycoengineered antibodies. In particular, peaks at m/z 1339, 1543 and 1705 correspond to nonfucosylated, complex oligosaccharides, which represent over 70% of the total oligosaccharides.

19. FIG. 3 of Exhibit D depicts the binding to FcγRIII receptors achieved by the glycoengineered antibodies compared to the nonglycoengineered antibodies. Substantially increased binding to FcγRIII receptors was observed for the glycoengineered antibody.

20. FIG. 4 of ExhibitD depicts the increased antibody-dependent cellular cytotoxicity observed for a glycoengineered antibody with increased levels of nonfucosylated Fc-region oligosaccharides compared to the nonglycoengineered antibody.

21. Thus, the attached Exhibits show that anti-CD20 antibodies coexpressed either with wild-type hManII or wild-type GnT-III or wild-type GnTIII *and* wild-type ManII have substantially increased affinity for the FcγRIIIA receptor and enhanced ADCC activity compared to the corresponding nonglycoengineered antibody. This demonstrates that the techniques taught in the present application can be used to produce glycoengineered recombinant antibodies having increased Fc-mediated cellular cytotoxicity (e.g., ADCC activity) and increased binding affinity for Fc receptors.

22. The undersigned further declares that all statements made herein of his knowledge are true, and that all statements made on information and belief are believed to be true, and that these statements were made with the knowledge that willful false statements or the like so made are punishable by fine, imprisonment, or both under § 1001 of Title 18, United States Code, and that such willful false statements may jeopardize the validity of any patent application or patent issued thereon.

August 20, 2006
Date

Pablo Umaña, Ph.D.

Supplemental Declaration for Patent Application

Docket Number: 1975.0010005/TJS/T-M

As legal representative of named inventor, James E. Bailey, I hereby declare that:

My residence, mailing address, and citizenship are as stated below next to my name.

I believe James E. Bailey was an original, first and joint inventor of the subject matter that is claimed and for which a patent is sought on the invention entitled **Glycosylation Engineering of Antibodies for Improving Antibody-Dependent Cellular Cytotoxicity**, the specification of which is attached hereto unless the following box is checked:

X was filed on August 5, 2003;

as United States Application Number 10/633,697; and

was amended on August 5, 2003 and November 1, 2005 (if applicable).

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information that is material to patentability as defined in 37 C.F.R. § 1.56, including for continuation-in-part applications, material information which became available between the filing date of the prior application and the national or PCT international filing date of the continuation-in-part application.

I hereby claim foreign priority benefits under 35 U.S.C. § 119(a)-(d) or (f) or § 365(b) of any foreign application(s) for patent, inventor's or plant breeder's rights certificate(s), or § 365(a) of any PCT international application, which designated at least one country other than the United States of America, listed below, and have also identified below, by checking the box, any foreign application for patent, inventor's or plant breeder's rights certificate(s), or PCT international application having a filing date before that of the application on which priority is claimed.

Prior Foreign Applications(s):			Priority Claimed	
			☐ Yes	☐ No
(Application No.)	(Country)	(Day/Month/Year Filed)		
			☐ Yes	☐ No
(Application No.)	(Country)	(Day/Month/Year Filed)		

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I hereby declare that all statements made herein of my knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under 18 U.S.C. § 1001 and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

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Getting the glycosylation right: Implications for the biotechnology industry

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Glycosylation is the most extensive of all the posttranslational modifications, and has important functions in the secretion, antigenicity and clearance of glycoproteins. In recent years major advances have been made in the cloning of glycosyltransferase enzymes, in understanding the varied biological functions of carbohydrates, and in the accurate analysis of glycoprotein heterogeneity. In this review we discuss the impact of these advances on the choice of a recombinant host cell line, in optimizing cell culture processes, and in choosing the appropriate level of glycosylation analysis for each stage of product development.

Keywords: glycosylation, recombinant, antigenicity, clearance, review, carbohydrates

better understanding of glycoprotein biosynthetic pathways and oning of many of the key enzymes involved, together with rogress in assigning functions to specific carbohydrate structures, as provided the groundwork for exploitation of glycobiology ithin the biotechnology industry. The carbohydrate components f glycoproteins can play crucial roles in protein folding, oligomer ;sembly and secretion processes, and in the clearance of glycoroteins from the bloodstream. Certain carbohydrate structures ave also been found to be antigenic, and regulatory authorities ıch as the US Food and Drug Administration are demanding ıcreasingly sophisticated carbohydrate analyses as part of the roduct or process validation[1,2].

It is not the purpose of this review to provide a general update n advances in glycobiology, which have already been covered by :veral excellent reviews published on biosynthetic pathways[3–6], ıe biological properties of carbohydrates[7], and cellular influences n the glycosylation process[8–11]. Instead, this review will focus on ıe differences in carbohydrate structures that may arise from ıoosing alternative gene expression systems and culture condions, their physiological significance, and the level of carbohyrate analysis that is appropriate at each stage of product evelopment.

ligosaccharide structures found on glycoproteins

)ligosaccharides can attach to proteins in three ways (Fig.1): 1) Via an *N*-glycosidic bond to the *R*-group of an Asn residue ithin the consensus sequence Asn-X-Ser/Thr (*N*-glycosylation). ll mature *N*-linked glycan structures have a common core of lan$_3$GlcNAc$_2$, which can form part of simple oligomannose strucıres or be extensively modified by the addition of other residues ıch as fucose, galactose, and sialic acids. Hybrid structures also xist where one or more arms of the glycan are modified and the emaining arm(s) contain only mannose. (2) Via an *O*-glycosidic bond to the *R*-group of Ser or Thr (*O*-glycosylation). *O*-linked glycosylation is extensive in structural proteins such as proteoglycans. Small glycan structures can also be *O*-linked to the side chain of hydroxylysine or hydroxyproline. (3) Carbohydrates are also components of the glycophosphatidylinositol anchor used to secure some proteins to cell membranes.

The presence of these consensus sequences by no means guarantees their glycosylation. They show varying degrees of occupancy with oligosaccharides (macroheterogeneity) dependent on their position within the protein and its conformation, the host cell type used for expression, and its physiological status. These three factors also determine the extent of variation in the type of sugar residues found within each oligosaccharide (microheterogeneity).

Choice of expression system

Bacteria. Common bacterial expression systems such as *Escherichia coli* have no capacity to glycosylate proteins in either *N*- or *O*-linked conformations[12]. Although other bacterial strains such as *Neisseria meningitidis* have recently been shown to *O*-glycosylate certain of their endogenous proteins, the trisaccharide added is different from *O*-linked sugars found in eukaryotes[13].

Yeast. Hypermannosylation (the addition of a large number of mannose residues to the core oligosaccharide), is a common property of most yeast strains[14] and can compromise the efficacy of recombinant proteins such as the hepatitis B vaccine[15] (Table 1). But hypermannosylation can be prevented by expressing the polypeptide in mutant yeast strains (e.g., mnn-9 or the temperature-sensitive ngd-29) in which *N*-glycosylation is confined to core oligosaccharide residues with a limited mannose content (up to Man$_8$GlcNAc$_2$), resulting in more effective vaccines[15,16]. There is also evidence to suggest that different *O*-glycosylation sites are used by yeast and mammalian cells[17].

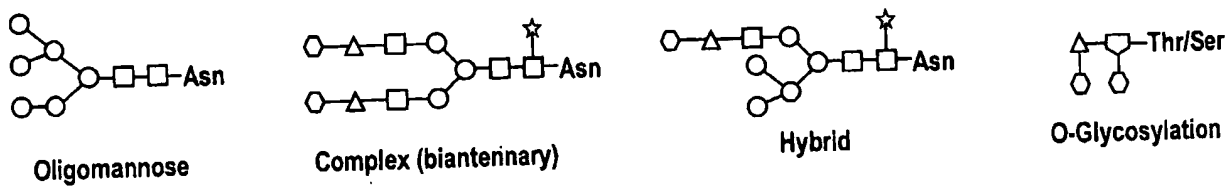


Figure 1. Common oligosaccharide structures found on glycoproteins: ○ Man, □ GlcNAc, △ Gal, ⬭ NeuAc, ☆ Fucose, ▽ GalNAc.

Plants. The few studies reporting the production of human proteins in plants have suggested that simple *N*-glycan structures that lack sialic acids are added, which may compromise activity. For example, erythropoietin (EPO) produced in tobacco cells has no biological activity in vivo, presumably because of its high clearance rate[18]. Another obstacle may be the presence of potentially allergenic residues such as core α1-3 linked fucose[19] or xylose β1-2 mannose[20].

Insects. The baculovirus-infected insect cell expression system has become a popular route for recombinant protein synthesis because of its short process development time and potentially high yields. Most evidence to date indicates that the *N*-glycosylation capabilities of this system are limited to producing only simple oligomannose-type oligosaccharides ($Man_{3-9}GlcNAc_2$)[21–23]; only a few studies have demonstrated complex *N*-linked glycans[24]. However, most of these data are derived from *Spodoptera frugiperda* (Sf9 and Sf21 lines), and other baculovirus-infected insect cell lines may differ. For example, the Ea4 line (derived from *Estigmena acrea*) can add some terminal galactose residues to recombinant interferon-γ[25], and secreted alkaline phosphatase produced in *Trichoplusia ni* (TN-368 and BTI-Tn-5B1-4 lines) has both galactose and terminal sialic acids[26].

Mammals. Species that are phylogenetically closer to humans may be expected to have more elements of the glycosylation machinery in common. Nevertheless, there are some surprising differences between the glycosylation characteristics of the rodent cell lines (routinely used for recombinant glycoprotein synthesis) and human tissues.

Mouse cells. Most mammals express the enzyme α1–3-galactosyltransferase, which generates Galα1,3-Galβ1,4-GlcNAc residues on membrane and secreted glycoproteins. The notable exceptions are in humans, apes, and Old World monkeys where the gene has become inactivated through frameshift mutations[27]. Certain mouse lines such as hybridomas, mouse-human heterohybridomas, and C127 cells synthesize some glycans terminating in Galα1,3-Galβ1,4-GlcNAc[28,29], particularly when grown in nonagitated flasks[30,31]. But other rodent lines such as mouse NS0 or rat Y0 myeloma, producing humanized antibodies, do not add Galα1,3-Galβ1,4-GlcNAc[32,33], and only induce a mild human immune response[34]. These residues are more likely to occur in hybridoma-derived antibodies where glycosylation occurs in the light chain, compared to the partially buried Fc glycosylation site[35]. Over 1% of

Table 1. Summary of the major glycosylation attributes for different cell expression systems.

		Type of Glycosylation				Saccharide Residues								
						Fucose		Galactose		Sialic acids				
Organism	Cell type	O-linked	Oligo-mannose	Hyper-mannose	Complex	α1-6 linked	α1-3 linked	Gal α1-3 Gal	SO_4-GalNAc	α2,6 linked	α2,3 linked	NeuGc	Bisecting GlcNAc	Glyco-sidases
Bacterium	*E. coli*	0	0	0	0	0	0	0	0	0	0	0	0	?
Yeast	*Sacromyces*	++	0	++++	0	0	0	0	0	0	0	0	0	?
Plant	Tobacco BY2	?	++	0	?	?	++	?	0	0	0	0	0	?
Insect	*S. frugiperda* Sf9	++	++++	0	D	++	+	0	0	0	0	0	0	++
	S. frugiperda Sf21	++	++++	0	D	++	?	?	?	D	D	?	0	?
	Trichoplusia ni	?	++	0	++	?	?	?	?	++	++	?	0	?
	E. acrea Ea4	?	+++	0	++	++	0	0	0	0	0	0	0	?
	M. brassicae	++	++++	0	0	++	?	?	?	?	?	?	0	?
Hamster	BHK	++	++	0	++	++	0	+	0	0	++	?	0	?
	CHO	++	++	0	++	++	D	0	0	0	++	+	0	++
Mouse	Hybridoma	++	++	0	++	++	0	++	0	0	++	+++	0	+
	Myeloma	++	++	0	++	++	0	++	0	+	+	+++	0	+
	C127	++	++	0	++	++	0	++	++	++	++	+++	0	?
	J558L	++	?	0	++	++	0	++	?	++	++	+++	0	?
	Transgenic	++	++	0	++	++	0	?	?	+	+	?	?	?
Rat	Y0 Myeloma	?	?	0	++	?	0	?	?	+	+	?	++	?
Goat & Sheep	Transgenic	?	++	0	++	++	0	?	?	+	+	?	?	?
Human	Liver	++	+	0	++	++	0	0	0	++	++	0	0	?
	Brain	++	++	0	++	++	++	0	0	++	++	0	+	?
	Pituitary	++	++	0	++	++	0	0	+++	+	+	0	++	?
	B-lymphocyte	++	0	0	+	++	0	0	0	+	+	0	++	?
	Namalwa	++	++	0	++	++	0	0	0	++	++	?	?	?
Human-Mouse	Heterohybridoma	?	++	0	++	++	0	0	0	+	+	+	0	?

Note that a few studies have analyzed the glycosylation profile of the same glycoprotein expressed in more than one system, therefore some characteristics may be protein-specific. Further details of individual cell lines are given in the text. 0=not detected; ?=not tested; D=disputed (conflicting results reported in different publications). + to ++++: an approximation of the levels of oligosaccharides detected.

human serum IgG is directed against the Gal(α1,3)-Gal-β1,4-GlcNAc epitope[36], which may a be consequence of its presence on enteric bacteria. Specific removal of this epitope from porcine endothelial cells substantially diminishes their reaction with the natural cytotoxic antibodies found in human serum[37], but comparisons of mouse C127 and chinese hamster ovary (CHO)-derived tissue plasminogen activator (tPA) reveal only minor differences in the pharmacokinetics induced by interaction with these antibodies[29].

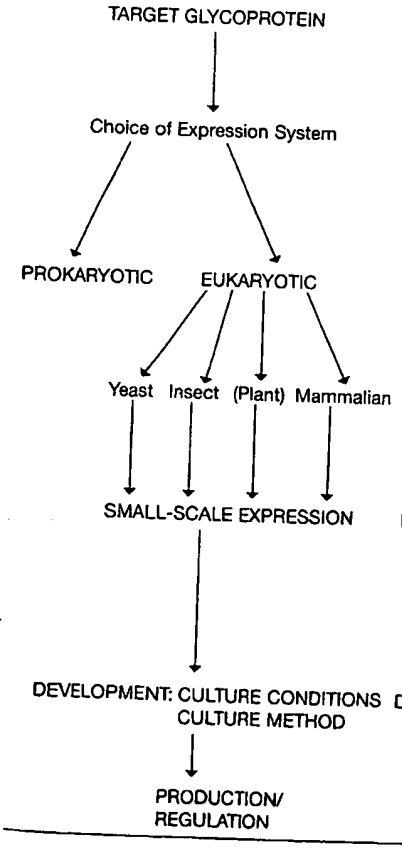
N-glycolylneuraminic acid (NeuGc) is a derivative of the sialic acid *N*-acetylneuraminic acid (NeuAc), and NeuGc levels have been shown to be more prevalent than NeuAc in antibodies derived from mouse or human-mouse hybridomas[38]. In contrast, glycoproteins in adult humans do not normally contain NeuGc, which is an oncofetal antigen[39]. Low levels of NeuGc (1% of total sialic acids) are tolerated in recombinant proteins such as EPO, but higher levels (e.g., in fetuin containing 7% NeuGc) can elicit an anti-NeuGc immune response[40]. Furthermore, high levels of terminal NeuGc on a chimeric CT4-IgG fusion protein are correlated with a rapid removal of the molecule from circulation, compared to the same protein bearing terminal NeuAc residues[41]. The hydroxylase enzyme that converts cytidine 5′-phosphate (CMP)-NeuAc to CMP-NeuGc has recently been cloned[42].

Hamster cells. Most of the CHO cell lines used for recombinant protein synthesis, such as Dux-B11, have fortuitously inactivated the gene for α1-3-galactosyltransferase[43] and make low levels of NeuGc (NeuAc is the dominant sialic acid found on CHO-derived glycoproteins)[44]. But cell lines such as CHO and baby hamster kidney (BHK) lack a functional α2,6-sialyltransferase (ST) enzyme, and synthesize exclusively α2,3-linked terminal sialic acids via α2,3-ST, in contrast to human and mouse cells, which have both enzymes[45]. Most rodent cells, including the CHO cell line, can be genetically modified to resemble the human glycan profile by transfection of appropriate glycosyltransferases. For example, the gene for rat α2,6-ST has been cloned into a recombinant CHO cell line to produce glycoproteins with both α2,6-linked and α2,3-linked NeuAc[45–47]. In addition, many mutants of CHO cells that display altered glycosylation properties that may prove useful hosts for expressing glycoproteins with minimal heterogeneity have been isolated[48].

Human cells. Other host cell lines have not been studied in sufficient depth to precisely define their glycosylation capabilities. Even the use of human host cell lines is not perfect, since the transformation event required in most cases to produce a stable cell line may itself result in altered glycosylation profiles[49]. However, the human lymphoblastoid Namalwa cell line performs *O*-linked and *N*-linked glycosylation efficiently, and preliminary studies on Namalwa-derived recombinant tPA have demonstrated all the human-type glycosylation characteristics[50,51]. All mouse-human heterohybridomas examined thus far have been found to follow the glycosylation characteristics of the mouse parental line[38,52].

Transgenic animals. Relatively few studies have been reported on the glycosylation of recombinant proteins expressed in the milk of transgenic animals. Those published from experiments in transgenic goats[53] suggest that a low level of complex glycans may be achieved. Furthermore, interferon-γ (IFN-γ) expressed in transgenic mice showed a greater proportion of truncated and oligomannose structures at the Asn_{97} site compared to IFN-γ that

Table 2. Decisions governing the type of glycosylation analyses appropriate at each stage of production.



		Question	Mono-saccharide composition analysis	Glyceran 'finger-printing'	Structural analysis/ epitope determination
DECISION 1	Q1.1 Is glycosylation necessary for the therapeutic profile of the target glycoprotein?	Q1.1	—	—	—
	Q1.2 If glycosylation does not occur, will underlying peptide regions be 'revealed' to the immune system?	Q1.2	—	—	—
DECISION 2	Q2.1 Are specific glycan structures required for full therapeutic activity?	Q2.1	✓	✓	✓
	Q2.2 Are certain glycan structures/epitopes to be avoided?	Q2.2	✓	✓	✓
	Q2.3 Is mammalian glycosylation preferred?	Q2.3	✓	✓	✓
DECISION 3	Q3.1 Is the anticipated glycosylation pattern obtained in practice?	Q3.1	—	✓	(✓)
	Q3.2 Is the glycosylation pattern obtained consistent with activity measurements?	Q3.2	—	—	—
	Q3.3 Is the glycosylation stable in the chosen formulation?	Q3.3	✓	✓	—
DECISION 4	Q4.1 Can a reproducible glycosylation pattern be obtained on a lot-to-lot basis?	Q4.1	✓	✓	(✓)
	Q4.2 What is the optimum method to monitor glycosylation?	Q4.2	✓	✓	(✓)
	Q4.3 What methods/levels of analysis will be submitted to regulatory authorities?	Q4.3	✓	✓	✓
	Lot-to-lot analysis on an on-going basis		✓	✓	—

expressed in CHO cells, although the level of glycosylation site occupancy was increased[21]. Glycoproteins can be remodelled in situ by the transgenic expression of extra glycosyltransferases in the mouse mammary gland[54].

Cells derived from specialized tissues. A significant proportion of IgG molecules produced by human B-lymphocytes possess a bisecting GlcNAc residue β1-4 linked to the central β-linked mannose of the core glycan. This residue may play a role in antibody-dependent cell-mediated cytotoxicity, and only certain rodent cell lines such as the rat Y0 myeloma (but not CHO cells or NS0 myelomas) produce recombinant antibodies containing this bisecting residue[55]. The GlcNAc transferase III enzyme which adds the residue has been cloned, thus, the opportunity exists for remodelling chimeric antibodies by transfecting this gene into host cells[55].

A large body of evidence suggests that natural IgG molecules lacking galactose are associated with rheumatoid arthritis[56]. This has also been tested experimentally by presenting the agalactosyl glycoforms of autoantibodies recognizing type II collagen in T-cell–primed mice, resulting in acute synovitis[57]. Thus, expression systems that result in a large proportion of recombinant therapeutic antibodies lacking galactose should be avoided. Low levels of sialic acids are typically found in both recombinant and natural antibodies[33], probably due to steric hindrance at the Fc glycosylation site.

Sulfated residues (SO_4-GalNAc-GlcNAc) appear on the outer arms of glycans attached to several pituitary glycoprotein hormones such as luteinizing hormone (LH), thyrotropin, proopiomelanocortin and certain proteases such as urokinase[58,59]. A specific GalNAc transferase and a terminal GalNAc sulfotransferase recognize special protein motifs in the nascent peptide (e.g., Pro-Leu-Arg) and are mainly restricted to the anterior pituitary gland. Therefore, only cell lines derived from the pituitary gland or endothelium (such as At20 and 293 cells) are able to perform this sulfation[60,61]. A hepatic receptor binds oligosaccharides terminating with SO_4-GalNAc residues, and could account for the rapid removal of sulfated LH from the circulation in contrast to the removal of follicle-stimulating hormone and chorionic gonadotropin, which bear terminal NeuAc residues[62]. Mouse C127 cells produce a different pattern of sulfation (NeuAc-α3SO_4-6Gal), which occurs after the addition of NeuAc[63].

The clearance of a given glycoprotein from the blood stream is highly dependent on its oligosaccharides, particularly those that are situated on the outer arms of the glycan structures[41]. Indeed, artificial glycosylation sites have even been introduced into small peptides in order to improve their pharmacokinetic properties or to render them resistant to blood proteases[64,65]. There are several receptors for specific oligosaccharide structures that contribute to the clearance of glycoproteins from the bloodstream, the most significant being the asialoglycoprotein receptor[66]. Other binding proteins recognize specific structures such as the Man-α6Man-β4GlcNAc-β4GlcNAc found in interleukin-6 (IL-6)[67], but their significance in the clearance of the majority of glycoproteins remains to be determined[68]. Studies in rats using EPO fractions enriched for particular glycoforms indicate that more highly branched glycans (e.g., triantennary or tetraantennary) are less susceptible to renal clearance than biantennary structures[69].

Cell culture conditions

Once the host cell line has been chosen, the cell culture conditions should be optimized in order to minimize glycoprotein heterogeneity and prevent deterioration of product quality during fermentation. Pioneering studies analyzing glycoproteins from batch samples[70,71] demonstrated that the culture environment can influence both the macroheterogeneity and microheterogeneity of oligosaccharides in recombinant glycoproteins[10,11,21].

Cell culture media. Monoclonal IgG_1 produced by mouse hybridomas in serum-free medium has higher levels of terminal sialic acid and galactose residues relative to that produced using serum[72], but antibody galactosylation is better in serum-containing medium for recombinant CHO cells[33]. Production of antibodies in cell culture results in more consistent glycosylation than achieved in ascites fluid[31,72,73]. Adaptation of BHK-21 cells producing a recombinant IL-2 mutant, from serum-containing to serum-free medium, results in substantial changes to its glycosylation, such as the complexity of glycan chains (number of arms, and higher levels of terminal sialylation and proximal α1-6 fucosylation), and increases the overall level of glycosylation[74].

The ambient glucose concentration affects the degree of glycosylation of monoclonal antibodies produced by human hybridomas in batch culture[75], and of IFN-γ produced by CHO cells in continuous culture[76,77]. Lipids such as dolichol act as key carriers in the glycosylation process, and lipid supplements alone or in combination with lipoprotein carriers can improve the *N*-glycosylation site occupancy of IFN-γ[78,79]. Work on hepatocytes suggests that provision of cytidine and uridine can also alter protein glycosylation by increasing the availability of nucleotide-sugars[80].

The application of sophisticated analytical techniques to analyze glycosylation microheterogeneity has led to some interesting findings. An increase in the percentage of oligomannose (predominantly $Man_5GlcNAc_2$) and truncated structures has been observed when monitoring both recombinant antibodies made by NS0 myelomas[32,81] and IFN-γ made by recombinant CHO cells[82]. In the former study, supplemental nutrients in fed-batch culture did not prevent this deterioration in glycosylation. In perfusion culture of BHK-21 cells producing a recombinant IL-2 mutant, nutrient limitations (glucose, amino acids, dO_2) led to short-term changes in macroheterogeneity, but microheterogeneity was largely unchanged[83].

Cell status. Lowering the protein synthetic rate by cycloheximide improves the glycosylation site occupancy of recombinant prolactin produced by C127 cells[84], but studies on tPA synthesis in CHO cells suggest that the rate of protein synthesis by itself has little influence on protein glycosylation[85]. Folding and disulfide bond formation certainly can influence the efficiency of *N*-linked glycosylation in some proteins. For example, low concentrations of the reducing agent dithiothreitol prevent cotranslational disulfide bond formation in the endoplasmic reticulum and lead to complete glycosylation of a tPA sequon that normally undergoes variable glycosylation[86]. Cell growth rate influences glycosyltransferase V levels in HepG2 cells[87]. Sodium butyrate is sometimes used to improve protein synthesis, but can change glycosylation by inducing a GlcNAc-transferase involved in *O*-glycosylation[88] and increasing sialyltransferase activity in recombinant CHO cells[89,90]. (Curiously, butyrate has the opposite effect in HepG2 cells[91].)

Bioreactor configuration. Mild hypoxia has minimal effects on the glycosylation of tPA produced by recombinant CHO cells[92], but influences the level of sialylation of recombinant FSH[89]. Similarly pH changes within the range 6.9–8.2 in the cell culture medium do not have a dramatic effect on the glycosylation profile of recombinant placental lactogen expressed in CHO cells, however there was some evidence for underglycosylation outside this range[93].

Increases in the concentration of ammonium ion in the culture medium above 2 mM may compromise sialyltransferase present in the Golgi, resulting in reduced α2,6-linked sialic acid in G-CSF produced by recombinant CHO cells[94]. Increased ammonium ions also reduced the extent of recombinant placental lactogen *N*-glycosylation by CHO cells, but this was dependent on the pH[95].

Product degradation. Various glycosidase activities have been measured in CHO cell lysates and culture supernatants, the most active of which is sialidase at neutral pH[96,97]. The soluble enzyme has been purified from culture supernatant of CHO cells[98] and can degrade glycans from proteins such as recombinant gp120. The CHO cell gene coding for this soluble sialidase was subsequently cloned and showed structural similarities to bacterial sialidases[99]. Although CHO cells also produce an α-*L*-fucosidase, this enzyme is incapable of releasing core α1,6-fucose from intact recombinant glycoproteins (gp120 or CD4), or the more peripheral Fuc-α1,3-GlcNAc linkage from serum α_1-acid glycoprotein[100]. Sialidase, β-galactosidase, β-hexosaminidase, and fucosidase can be detected at low levels in supernatants from mouse 293, NS0, and hybridoma cells, and the sialidase activity is much lower than that found in CHO cells[101].

In a contrasting study, purified monoclonal antibody was incubated with supernatants from various mammalian cell lines (CHO K1, BHK-21, mouse C127, P3-X63, Ag8.653, and a human-mouse heterohybridoma) and an insect cell line (SF-21AE), and only the insect line showed evidence of glycosidase activity in the supernatant[102].

Controlled carbohydrate modification. Advances are being made in the chemical production of oligosaccharides (e.g., synthesis of the core *N*-glycan structure $Man_3GlcNAc_2$ and oligomannose glycans from monosaccharides has recently been accomplished[103,104]). This opens the possibility of adding defined glycan structures after recombinant protein synthesis and secretion. In addition, the cloning and expression in *E. coli* of several key glycosyltransferases will facilitate the postharvest remodelling of glycoproteins produced in cell culture[105,106], making them more acceptable as human therapeutic proteins.

Conclusions

Improvements in glycosylation analysis have enabled scientists to judge how the glycan structures of recombinant glycoproteins compare to their natural human counterparts. Ultimately, production may go beyond the reiteration of the human glycosylation profile, because it may be desirable to alter the product's bioactivity or pharmacokinetics in vivo by altering specific glycan structures. The initial choice of expression system will continue to be of crucial importance, and as more recombinant proteins are expressed in different cell lines a pattern of glycoform predictions can be assembled (Table 1), although these conclusions must remain speculative until more data are available. The influences of the cell culture process (including the effects of scale-up) are not as well defined at present, but as more studies are published generic protocols may emerge to produce consistent (albeit heterogeneous) glycosylation patterns. A more difficult objective for cell technology will be to produce a single invariant protein glycoform, rather than the current mixture of glycoforms each bearing different characteristics (analogous to chiral separation of small molecule drugs).

An increased awareness of the importance of product glycosylation has lead to more detailed carbohydrate analysis at earlier stages of product development than in the past. The choice of a particular cell expression system, and the influence of a different manufacturing process on the biochemical consistency of the product are now being evaluated (Table 2). A small but increasing number of glycosylation analyses are requested by legal departments, who may use the resulting information in patent applications to substantiate claims and to distinguish their organization's material from that of their competitors.

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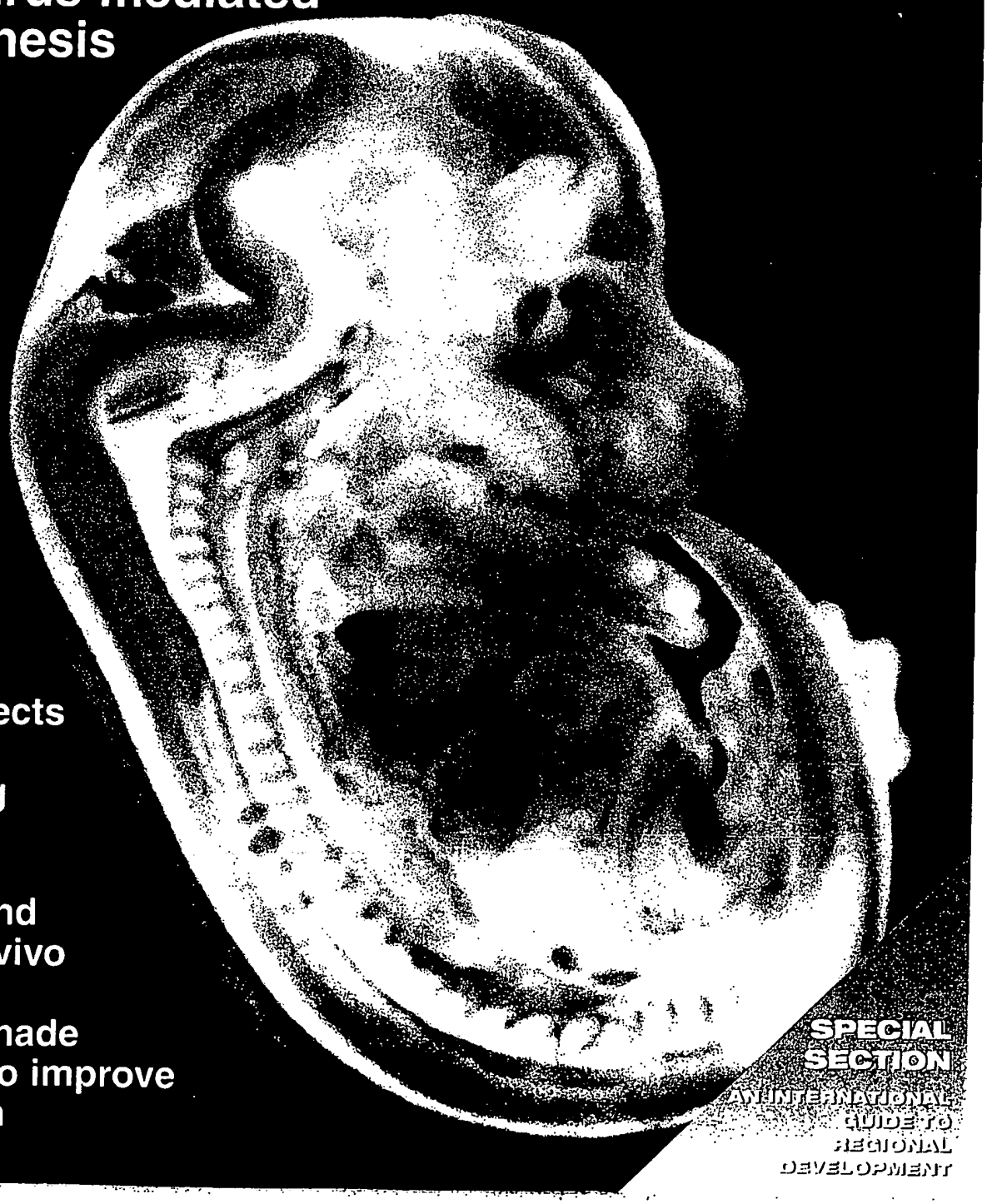

Chilling effects of biolipid engineering

Nitric oxide detection and imaging in vivo

Disabling shade avoidance to improve crop growth

SPECIAL SECTION
AN INTERNATIONAL GUIDE TO REGIONAL DEVELOPMENT

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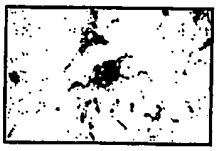

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RESEARCH REVIEW

RESEARCH ARTICLES

BPA INTERNATIONAL

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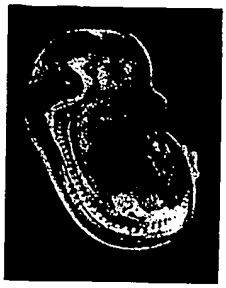

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The cover. Section of a mouse embryo expressing an adenovirus-mediated lacZ transgene, shown as ubiquitous blue staining. Details can be found on pp. 942 and 982.

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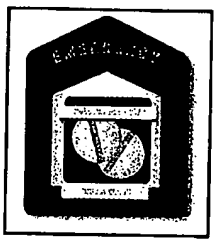
OPINION

IN BRIEF



ANALYSIS

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RESOURCES

CLASSIFIED

REVIEW/SYNTHÈSE

Biosynthetic controls that determine the branching and microheterogeneity of protein-bound oligosaccharides[1]

HARRY SCHACHTER
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Received October 18, 1985

Schachter, H. (1986) Biosynthetic controls that determine the branching and microheterogeneity of protein-bound oligosaccharides. *Biochem. Cell Biol. 64*, 163–181

Detailed studies on the enzyme machinery responsible for the biosynthesis of protein-bound oligosaccharides of the Asn-GlcNAc and Ser(Thr)-GalNAc linkage types have allowed the formulation of some general rules which explain, at least in part, the branching patterns and microheterogeneity of these structures. These rules are discussed under the following headings: (*i*) competition of two or more enzymes for a common substrate; (*ii*) controls at the level of enzyme substrate specificity (e.g., critical sugar residues which turn enzyme activity on or off, branch specificity, and the role of the polypeptide in the glycoprotein substrate); (*iii*) substrate availability.

Schachter, H. (1986) Biosynthetic controls that determine the branching and microheterogeneity of protein-bound oligosaccharides. *Biochem. Cell Biol. 64*, 163–181

Les études détaillées de la machinerie enzymatique responsable de la biosynthèse des oligosaccharides liés aux protéines par des liaisons du type Asn-GlcNAc et Ser(Thr)-GalNAc ont permis la formulation de certaines règles générales qui expliquent, du moins en partie, les profils de ramification et la microhétérogénéité de ces structures. Nous discutons de ces règles sous les rubriques suivantes : (*i*) compétition de deux enzymes ou plus pour un substrat commun; (*ii*) contrôles au niveau de la spécificité enzymatique à l'égard des substrats (p. ex., résidus glucidiques critiques qui amorcent ou qui arrêtent l'activité enzymatique, spécificité des ramifications, rôle du polypeptide dans le substrat glycoprotéique); (*iii*) disponibilité des substrats.

[Traduit par la revue]

Introduction

Protein-bound oligosaccharides fall into several classes depending on the linkage between sugar and amino acid. The classes to be discussed in this review are of the Asn-GlcNAc (*N*-glycosidic linkage) and Ser(Thr)-GalNac (*O*-glycosidic linkage) types. Modern analytical techniques have recently led to the elucidation of literally hundreds of different protein-bound oligosaccharides. This work has shown that two properties distinguish complex carbohydrates from proteins and nucleic acids, the other two classes of biological macromolecules, namely (*i*) branching and (*ii*) microheterogencity. These properties pose both challenges and problems for researchers interested in the function and biosynthesis of complex carbohydrates.

The functions of complex carbohydrates are not properly understood and will not be discussed in detail. Several different functions have been identified (1). (*i*) The presence or absence of different oligosaccharides can modulate the physicochemical properties of a glycoprotein, e.g., solubility, viscosity, charge, and denaturation. (*ii*) Carbohydrate may protect the polypeptide moiety of a glycoprotein against uncontrolled proteolysis, both inside and outside the cell. (*iii*) Some glycoproteins undergo proteolytic processing within the cell from a large primary translation product to smaller final products. This process can be affected by the presence of protein-bound carbohydrate. (*iv*) Although carbohydrate is not usually involved in the biological activity of biologically active glycoproteins, there are important exceptions, e.g., human chorionic gonadotropin. (*v*) Some glycoproteins require proper glycosylation for their insertion into membrane, for secretion, or for proper intracellular migration and sorting. (*vi*) Glycosylation has been implicated in embryonic development and differentiation.

We are only beginning to understand some of these

ABBREVIATIONS: Gn or GlcNAc, *N*-acetylglucosamine; GN or GalNac, *N*-acetylgalactosamine; (Gn), bisecting GlcNAc; G, or Gal, galactose; M or Man, mannose; F or Fuc, fucose; S, sialic acid; Gn-T or GlcNAc-T, *N*-acetylglucosaminyltransferase; G-T or Gal-T, galactosyltransferase; αMase II, α-mannosidase II; F-T or Fuc-T, fucosyltransferase; S-T or sialyl-T, sialyltransferase; IgG, immunoglobulin G; H, heavy; L, light; NMR, nuclear magnetic resonance.

[1]This talk was given at the presentation of the Boehringer Mannheim Canada Prize of the Canadian Biochemical Society, on June 18, 1985.

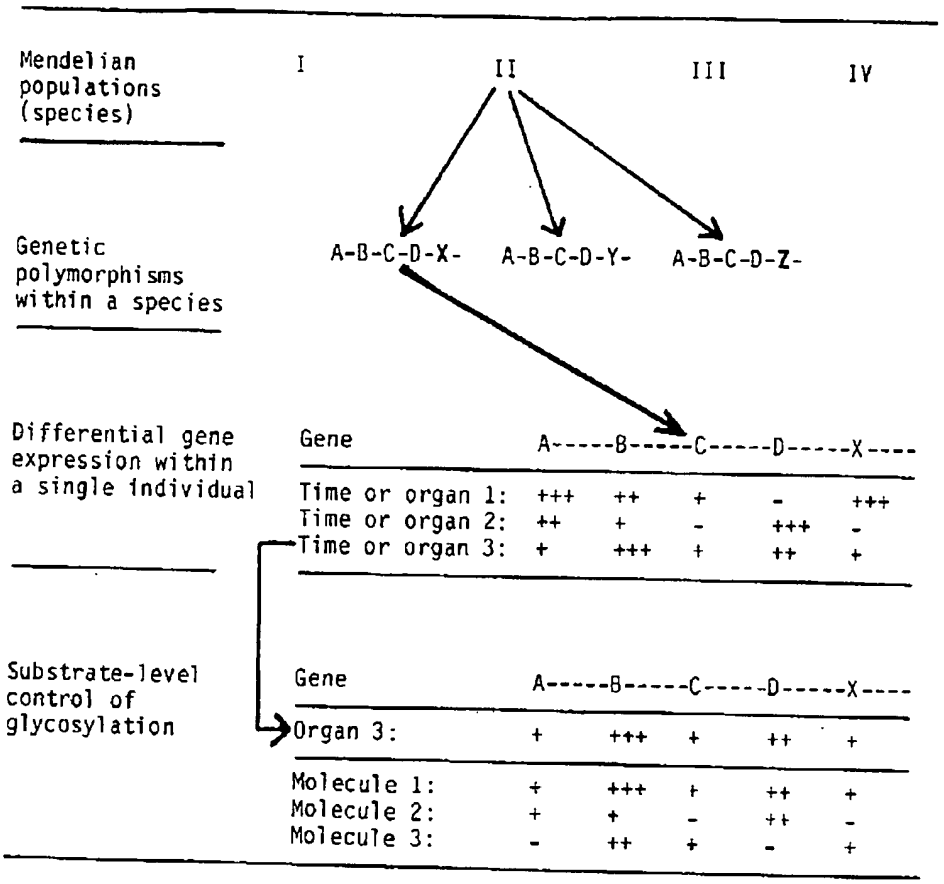


FIG. 1. Hierarchies of control involved in complex carbohydrate assembly. Species differ in the genes controlling glycosylation. Within a single species, there is genetic polymorphism (genes, X, Y, and Z in the above scheme may, for example, respectively, code for the blood group A-dependent α3-Gal-transferase, the blood group B-dependent α3-Gal-transferase, or the absence of either transferase). Differential gene expression between organs or at different times in the same organ occurs during differentiation. Even in a single organ at a particular time, substrate-level factors may modify the activity of a glycosyltransferase.

functions in higher organisms, e.g., the role of cell surface carbohydrates in the interactions of cells with their cellular and fluid environments. There is preliminary evidence that the branching patterns of cell surface carbohydrate may be important in oncogenic transformation (2–5). However, the oligosaccharides on some glycoproteins show extreme microheterogeneity: e.g., highly purified hen ovomucoid has more than 20 different oligosaccharides (6, 7). This phenomenon is common and is difficult to reconcile with the hypothesis that complex carbohydrates mediate an information-transfer role.

The highly branched nature of complex carbohydrates has important implications conerning their synthesis. Proteins and nucleic acids are linear molecules and are synthesized by a highly accurate template mechanism; DNA acts as a template for RNA and RNA acts as a template for protein. Because they are branched, complex carbohydrates cannot be made in this way. Genetic information is transferred via an indirect **nontemplate** pathway. Genes code for the protein backbone of the glycoprotein, for the glycosyltransferases and glycosidases that form the oligosaccharides, for substrate and cofactor availability, and for the construction of the endomembrane assembly lines within which all complex carbohydrates are synthesized. The end result is that nucleic acid and protein assembly tends to be very accurate, while the assembly of complex carbohydrates is prone to variation and leads to the phenomenon of microheterogeneity. It is not known whether this variation is random and serves little purpose or whether microheterogeneity and the large diversity of structures have a function not yet understood.

Biosynthetic factors involved in complex carbohydrate microheterogeneity

Figure 1 attempts to outline the various factors which contribute to the microheterogeneity of complex carbohydrates.

TABLE 1. Tissue and species survey for Gn-T*

Enzyme source	Gn-T activity (nmol/h per milligram) Core 3 β3-Gn-T	Core 4 β6-Gn-T	Core 2 β6-Gn-T	2/4 ratio	Elongation β3-Gn-T
Rat					
Colon	19.7	108	135	1.3	17
Stomach	0.4	25.2	29.6	1.2	1
Submaxillary gland	0	4.1	4.4	1.1	0
Small intestine	3.0	38.6	48.6	1.3	1
Liver microsomes	1.0	<0.6	<0.6	—	<0.4
Pig					
Colon	20.5	13.4	51.2	3.8	14
Stomach	0.8	167	334	2.0	9
Submaxillary gland	0	0	0	—	0
Dog					
Submaxillary gland	0	17.8	114	6.4	0
Monkey					
Colon	2.4	4.7	11.9	2.5	—
Stomach	0.4	9.5	25.9	2.7	—
Human					
Colon	5.5	10.3	24.0	2.3	0–9.6
Serum	—	—	—	—	0
Sheep					
Stomach	0.6	16.9	21.3	1.3	—

*The enzyme reactions are as follows. Core 3 β3-Gn-T (15, 16): UDP-GlcNAc + GalNAc-R → GlcNAcβ1-3GalNAc-R + UDP. Core 4 β6-Gn-T (15, 16): UDP-GlcNAc + GlcNAcβ1-3GalNAc-R → GlcNAcβ1-3(GlcNAcβ1-6)GalNAc-R + UDP. Core 2 β6-Gn-T (17, 18): UDP-GlcNAc + Galβ1-3GalNAc-R → Galβ1-3(GlcNAcβ1-6)GalNAc-R + UDP. Elongation β3-Gn-T (19, 20): UDP-GlcNAc + Galβ1-3(Rβ1-6)GalNAc-R → GlcNAcβ1-3Galβ1-3(Rβ1-6)GalNAc-R + UDP.

Mendelian populations (or species)

Sufficient data is now available to indicate that species may differ in the nature of their complex carbohydrates. For example, Kobata and his colleagues detected species-specific differences when they compared the carbohydrate structures present on γ-glutamyl transpeptidase purified from the kidney and liver of rats, mice, and cows (2, 4, 8–12), on fibronectin from bovine and human plasma, and on α_1-acid glycoprotein from rat and human plasma (13, 14). We have observed similar species-specific differences in the activities of glycosyltransferases involved in the assembly of the core structures of the *O*-glycosidically linked oligosaccharides found in mucin-type glycoproteins (Table 1; Ref. 15). The functional significance of these findings is not clear.

Genetic polymorphism within a species

Individuals within a Mendelian population, and even within a subspecies, can differ in their genetic makeup. Only true clones obtained by asexual reproduction are genetically identical. The best example of genetic polymorphism in man is due to complex carbohydrates, i.e., the ABO and Lewis human blood group antigens. The genes responsible for these antigens code for a series of glycosyltransferases (A-dependent α3-GalNAc-transferase, B-dependent α3-Gal-transferase, H-dependent α2-fucosyltransferase, and Lewis-dependent α4-fucosyltransferase).

There are two major forces for the above type of genetic variability: (*i*) mutation and recombination due to sexual matings followed by natural selection and (*ii*) genetic drift due to random or adaptively neutral loss of genes in sexual matings. It is interesting that not only are many apparently adaptively neutral genetic polymorphisms preserved but, in fact, some decidedly harmful polymorphisms are also preserved, e.g., sickle cell anemia. One possible reason is the concept of balanced polymorphism due to hybrid vigour, i.e., that the heterozygote has a higher survival potential than either homozygote. In the case of sickle cell anemia, the heterozygote survives malaria better than normal homozygotes. Natural selection ensures optimum survival of the species, not of the individual.

The lesson to be learned is that the survival of certain molecules through evolution may be due to a relatively

minor and undetectable selective advantage. A large amount of ATP is required to synthesize a complex carbohydrate. This implies that the organism probably derives some advantage from evolutionary preservation of at least some of these large and complex structures.

Differential gene expression within an individual

The development of the fertilized egg into a complex organism (embryogenesis) requires organ development and differentiation. This process involves quantitative and qualitative differences in gene expression rates between different organs at any one time and in a particular organ at different times. Although the controls involved in eukaryotes are not yet established, it is clear that the environment of a cell must play a role during differentiation by influencing which genes are turned off and which are turned on.

There are several lines of evidence to suggest that complex carbohydrates on the cell surface are involved in the embryogenesis and differentiation processes. Hematopoietic tissue involves the differentiation of pluripotent stem cells into T and B lymphocytes, erythrocytes, granulocytes, platelets, etc. Marked changes have been observed in glycoprotein and glycolipid patterns during both erythropoiesis and granulocyte (myeloid cell) differentiation (21–27). For example, human foetal erythrocytes carry the blood group *i* antigen which is a linear oligosaccharide consisting of Galβ1-4GlcNAcβ1-3 repeating units, whereas most adults carry on their red cells the I antigen containing the branched Galβ1-4GlcNAcβ1-3(Galβ1-4GlcNAcβ1-6)-Gal-structure. Narasimhan and colleagues (Ref. 28 and unpublished data) have observed that human B cell lineage lymphoma lines involved in immunoglobulin synthesis contain GlcNAc-transferase III (29), the enzyme which inserts a bisecting GlcNAc in β1-4 linkage to the β-linked Man of the core of *N*-glycosyl oligosaccharides; resting T and B lymphocytes and T-lineage acute lymphatic leukemia cells were essentially devoid of GlcNAc-transferase III activity. Changes in complex carbohydrate structure have also been observed as embryogenesis progresses (23, 24, 30–32).

Changes in the expression of complex carbohydrate antigens have been detected during oncogenesis. These tumour-associated antigens often represent retrogenetic expression of carbohydrate synthesis to a particular stage of foetal development and are called oncofoetal antigens. It is therefore not surprising that these antigens are not tumour-specific, since they are synthesized in normal foetal tissues (23, 24).

Several studies have shown differential gene expression between different organs for complex carbohydrate synthesis. Kobata and colleagues observed consistent differences between kidney and liver in their studies on γ-glutamyl transpeptidase (2, 4, 8–12). The most dramatic difference was the presence of bisecting GlcNAc residues (linked β1-4 to the β-linked Man of *N*-glycosyl oligosaccharide cores) in the enzyme from rat, cow, and mouse kidney but the complete absence of this residue from rat and mouse liver enzyme. Rat hepatoma enzyme, however, showed the presence of bisecting GlcNAc residues. We have also observed organ-specific differences in our studies on the synthesis of mucin cores (Table 1; Ref. 15). For example, the activity of β3-GlcNAc-transferase responsible for synthesis of core type 3 (GlcNAcβ1-3GalNAc) is high in the rat, pig, monkey, and human colon but is very low in the stomach and submaxillary gland.

Endomembrane factors

Even in a particular organ at a particular time, a polypeptide core may acquire different types of oligosaccharide; i.e., the individual molecules of a so-called pure glycoprotein preparation will have identical amino acid sequences but yet will differ from one another in oligosaccharide side chains (e.g., α_1-acid glycoprotein, hen ovalbumin, hen ovomucoid, transferrin, etc). Or, a single organ may make two glycoproteins with totally different oligosaccharides: e.g., the hen oviduct (6, 7, 33) makes ovalbumin with high mannose and bisected hybrid *N*-glycosyl oligosaccharides but ovomucoid, in the same organ, ends up with truncated (primarily GlcNAc-terminal antennae) bisected highly branched complex *N*-glycosyl oligosaccharides (Fig. 2).

A relatively simple explanation for microheterogeneity is that polypeptides travel through different endomembrane assembly lines, either in the same cell or in different cells. This explanation applies both to the situation in which different oligosaccharides appear at a particular amino acid position of a particular polypeptide chain and to the situation in which a single organ makes different polypeptides with different oligosaccharides. This hypothesis requires a mechanism for sorting peptides to the different assembly lines. If all molecules made by a particular organ move through identical assembly lines, then factors operative at the endomembrane level must be responsible for microheterogeneity. The remainder of this review will deal with these endomembrane or "substrate-level" factors.

The endomembrane assembly line

A great deal of information is now available on the structures of *N*- and *O*-glycosyl oligosaccharides (14, 34, 35) and on the detailed enzymatic steps involved in their synthesis (35–40). Oligosaccharide synthesis occurs on a membranous assembly line. The polypeptide backbones of all glycoproteins are synthesized on membrane-bound polyribosomes and all carbohydrate addition occurs on the lumenal side of the endomembrane system.

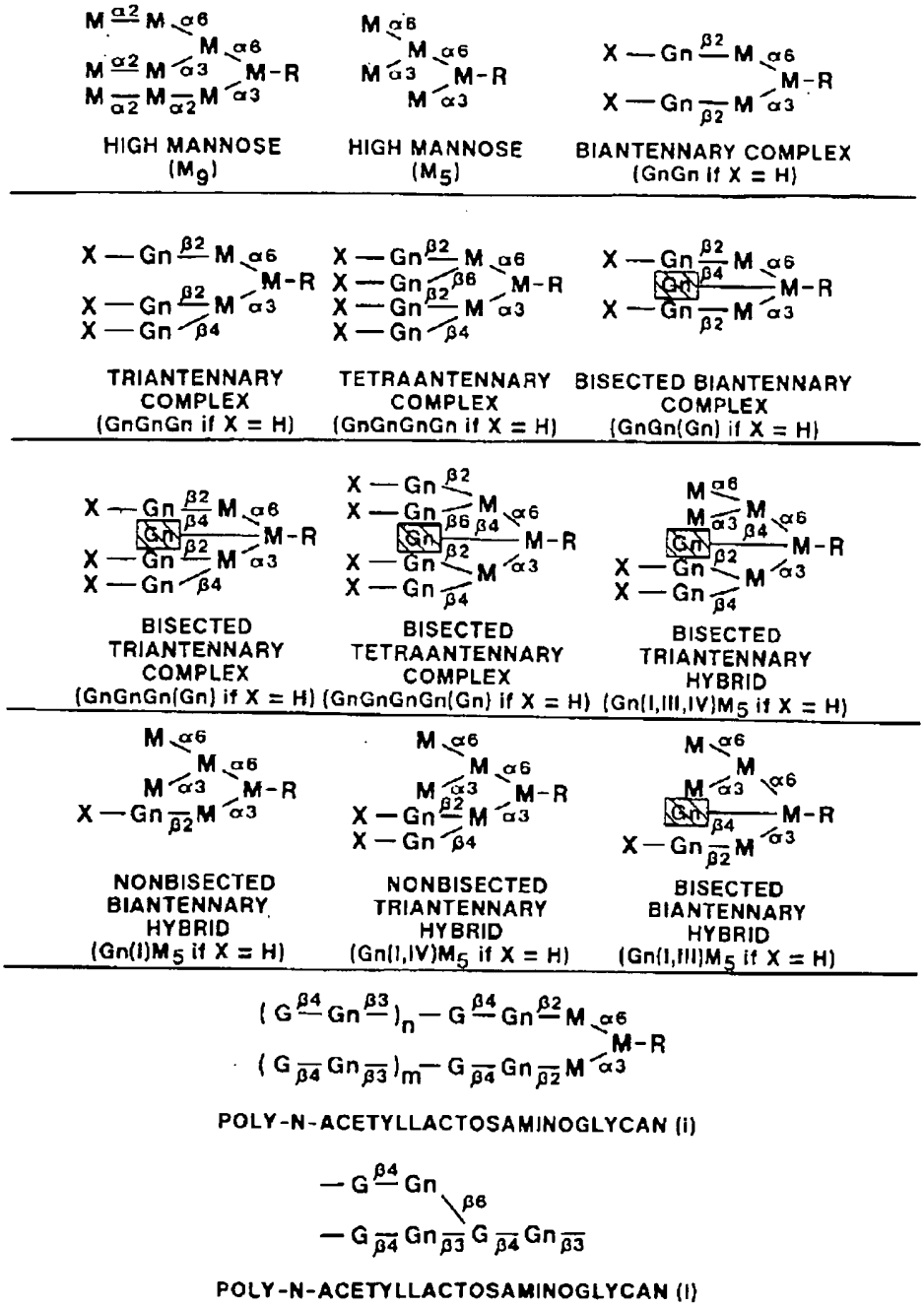


FIG. 2. Examples of *N*-glycosyl oligosaccharides (OS). High mannose OS are named according to the number of Man (M) residues, e.g., M_5. Complex OS are named according to the sugars at the nonreducing termini, with the Manα1-6 arm being named first (M, Man; Gn, GlcNAc; G, Gal; (Gn), bisecting GlcNAc). Hybrid OS are named as shown, using the numbering system in Fig. 6 to designate the arms. X can be H, Galβ1-4(3), or sialylα2-3(6)Galβ1-4(3).

Figure 3, taken form the excellent review by Kornfeld and Kornfeld (35), outlines the basic steps in the assembly of biantennary *N*-glycosyl oligosaccharides. Synthesis starts in the rough endoplasmic reticulum with the transfer of a large oligosaccharide from dolichol pyrophosphate oligosaccharide to an asparagine residue in the polypeptide backbone (step 1, Fig. 3). This is followed by oligosaccharide processing within both the rough endoplasmic reticulum (steps 2 to 4, Fig. 3) and Golgi apparatus (steps 5 and 7, Fig. 3) as the glycoprotein moves through the endomembrane assembly line.

Our laboratory has been concerned primarily with the mechanisms which determine the branching patterns of complex and hybrid *N*-glycosyl oligosaccharides (Fig.

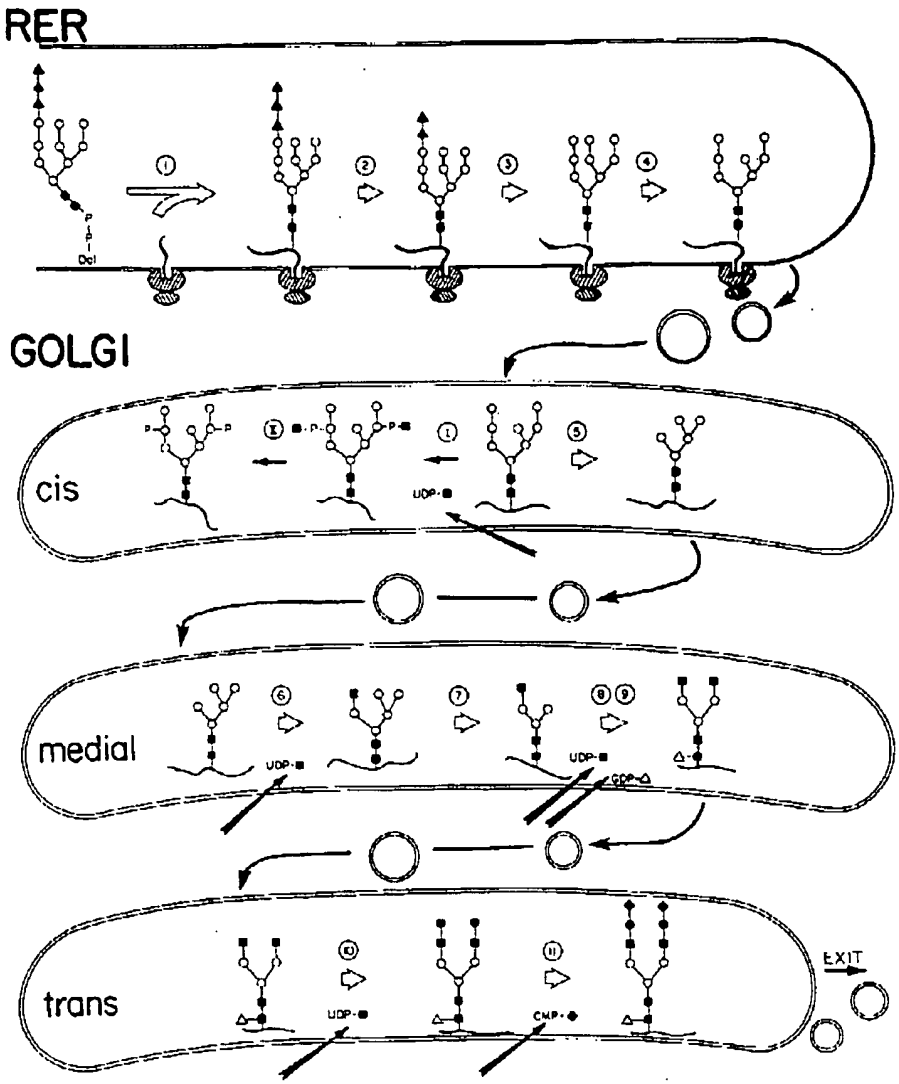


FIG. 3. Biosynthesis of *N*-glycosyl nonbisected biantennary complex oligosaccharide (from Ref. 35). Processing enzymes: (1) transfer of $Glc_3Man_9GlcNAc_2$ from dolichol pyrophosphate oligosaccharide to peptide nascently bound to ribosome; (2) α-glucosidase I; (3) α-glucosidase II; (4) rough endoplasmic reticulum (RER) α2-mannosidase; (5) α-mannosidase I; (6) GlcNAc-transferase I; (7) α-mannosidase II; (8) GlcNAc-transferase II; (9) α6-fucosyltransferase; (10) Gal-transferase; (11) sialyltransferase; (I) *N*-acetylglucosaminyl phosphotransferase, which acts on hydrolases destined for the lysosomes; (II) *N*-acetylglucosamine-1-phosphodiester α-*N*-acetylglucosaminidase, which exposes the Man-6-phosphate signal required for movement of hydrolases to the lysosome. Transport of nucleotide sugars into the lumen is indicated. ■, GlcNAc; ○, Man; ▲, Glc; △, Fuc; ●, Gal; ◆, sialic acid.

2) and with the synthesis of the four major core types of *O*-glycosyl oligosaccharides (Fig. 4). In particular, as mentioned in the previous section, we have been interested in the "substrate-level" controls which operate within the endomembrane system to produce the large variety of oligosaccharides that occur on mammalian and avian glycoproteins. These steps occur within the Golgi apparatus.

The Golgi apparatus is now believed to be subdivided into subcompartments with different functions (Fig. 3; Refs. 35, 41, and 42). The steps involved in assembly of the two antennae of a typical biantennary complex *N*-glycosyl oligosaccharide probably occur in the medial and trans Golgi compartments (steps 6 and 8–11, Fig. 3). Although Fig. 3 transmits a great deal of information in a clear and concise manner, it is quite incomplete since it lacks the pathways for the synthesis of *N*-glycosyl oligosaccharides of the hybrid type and of complex types larger than biantennary. An expansion of the synthetic scheme which includes the latter structures is shown in Fig. 5. It is necessary to use a shorthand notation to fit all the pathways into a single page and the

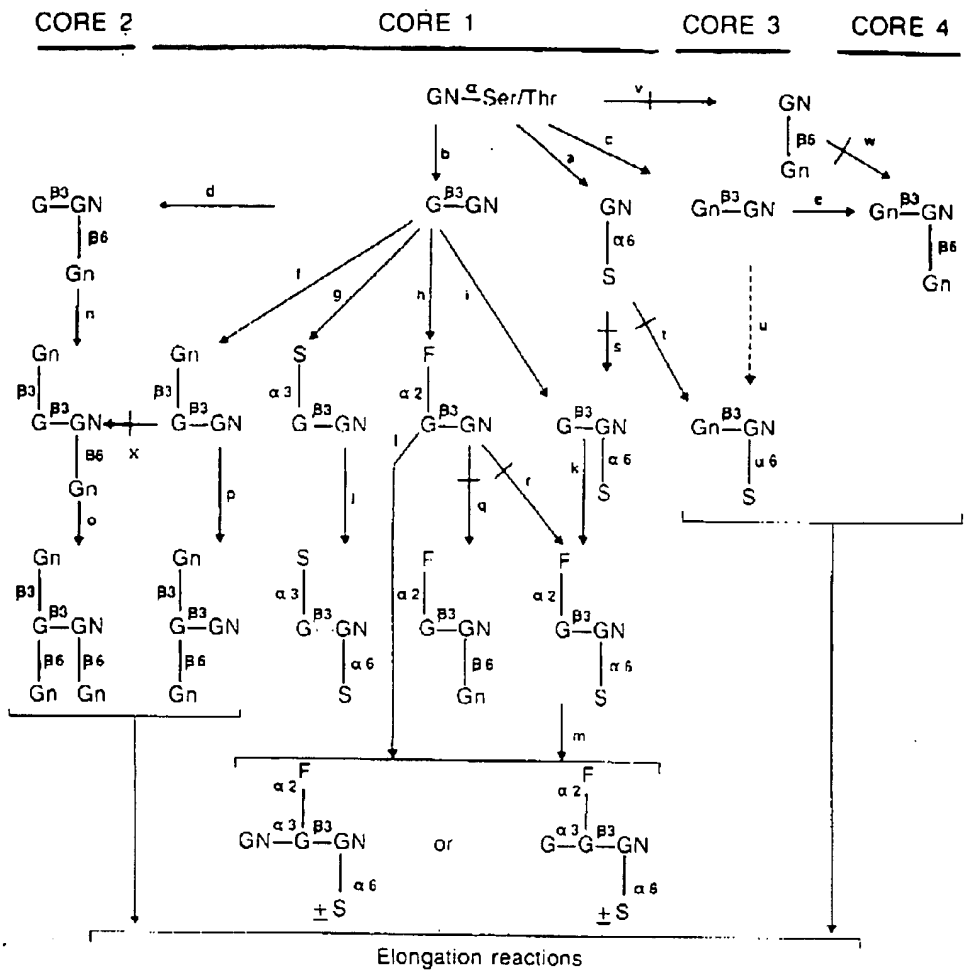


FIG. 4. Biosynthesis of *O*-glycosyl oligosaccharides. Four major core classes are shown: core 1 Galβ1-3GalNAc, core 2 Galβ1-3(GlcNAcβ1-6)GalNAc, core 3 GlcNAcβ1-3GalNAc, and core 4 GlcNAcβ1-3(GlcNAcβ1-6)GalNAc. GN, GalNAc; Gn, GlcNAc; G, Gal; S, sialic acid; F, Fuc. All steps except *u* have been proven by *in vitro* experiments (Refs. 15, 16, 19, 20, 37 and 40; I. Brockhausen, A. Koenderman, K. L. Matta, D. H. Van den Eijnden, and H. Schachter, in preparation).

visual impact is not as clear as the diagrams used in Fig. 3. Even Fig. 5 is not complete since it omits GlcNAc-transferases V, VI, and VII (Fig. 6).

Control of glycoprotein synthesis at the substrate level

Table 2 lists the various mechanisms which control the assembly of protein-bound oligosaccharide at the substrate level.

Competition for a common substrate

At many points in the synthetic schemes for both *N*- and *O*-glycosyl oligosaccharides (Figs. 4 and 5), a particular substrate can be acted on by more than one enzyme. Although the schemes shown in Figs. 4 and 5 are composite schemes based on *in vitro* assays carried out in different tissues, it is assumed that they approximate the *in vivo* situation and that competition for a common substrate will also occur *in vivo*.

Several important "crossroads" can be pointed out (Fig. 5). $Gn(I)M_5$ can be acted on by (*i*) GlcNAc-transferase III to form bisected biantennary five-Man hybrids (43), (*ii*) GlcNAc-transferase IV to form non-bisected triantennary five-Man hybrids (43), (*iii*) α6-fucosyltransferase (44), or (*iv*) α-mannosidase II to form MGn, or (*v*) can move from the medial to the trans Golgi to form nonbisected biantennary five-Man hybrids. Once the bisecting GlcNAc has been inserted by GlcNAc-transferase III, α-mannosidase II can no longer act (45) and the pathway is fixed into the production of hybrid structures (Fig. 5). The rules permit the formation of five-Man hybrid structures with a core α6-fucose

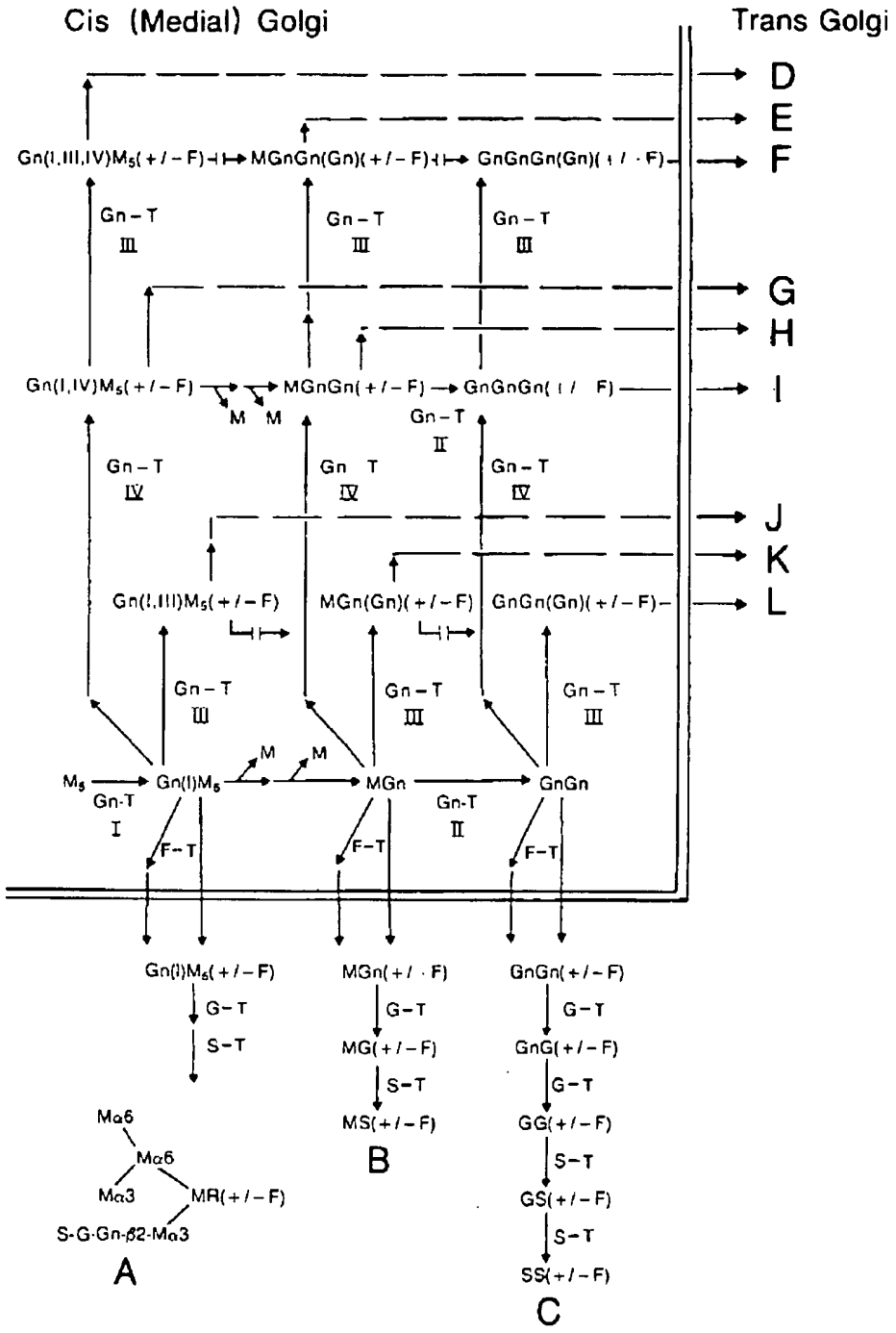


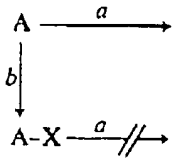
FIG. 5. Biosynthesis of *N*-glycosyl oligosaccharides (OS) showing the conversion of M_5 into the following. (A) Five-Man nonbisected biantennary hybrid OS; (B) three-Man nonbisected biantennary hybrid OS (or incomplete biantennary complex OS); (C) nonbisected biantennary complex OS; (D) five-Man bisected triantennary hybrid OS; (E) three-Man bisected triantennary hybrid OS (or incomplete bisected triantennary complex OS); (F) bisected triantennary complex OS; (G) five-Man nonbisected triantennary hybrid OS; (H) three-Man nonbisected triantennary hybrid OS (or incomplete triantennary complex OS); (I) nonbisected triantennary complex; (J) five-Man bisected biantennary hybrid OS; (K) three-Man bisected biantennary hybrid OS (or incomplete bisected biantennary complex OS); (L) bisected biantennary complex. M, Man; Gn, GlcNAc; G, Gal; S, sialic acid; (Gn), bisecting GlcNAc; F, Fuc; F-T, fucosyltransferase; Gn-T, GlcNAc-transferase; S-T, sialyltransferase; G-T, Gal-transferase.

TABLE 2. Control of glycoprotein synthesis at the substrate level

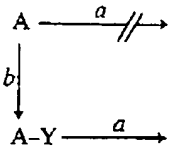
1. Competition for a common substrate

2. Substrate specificity of glycosyltransferases

(*i*) GO – NO GO



(*ii*) NO GO – GO



(*iii*) Recognition site distinct from catalytic site
(*iv*) Branch specificity
(*v*) Role of polypeptide

3. Substrate availability
   (*i*) Subcellular compartments as assembly lines
   (*ii*) Transport of nucleotide sugars across membranes

4. Other factors: pH, cations, phosphatases, glycosidases. Phospholipid environment

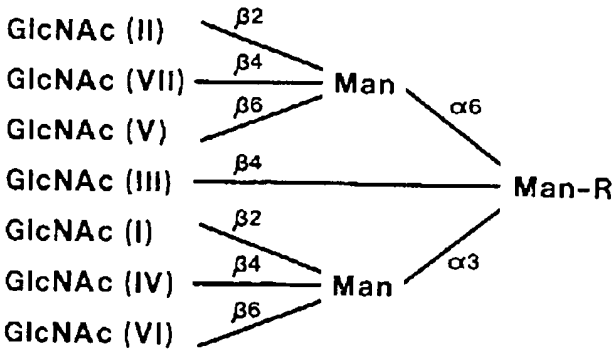


FIG. 6. A hypothetical structure showing all the antennary GlcNAc residues described to date. GlcNAc linked 1-3 to either arm of the core, 1-2 to the β-linked Man, or in α-linkages are theoretically possible, but have not yet been described. GlcNAc residues have been numbered arbitarily as indicated. This numbering system is used to name hybrid oligosaccharides (Fig. 2) and GlcNAc-transferases.

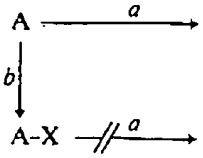
residue, but such structures have not as yet been reported.

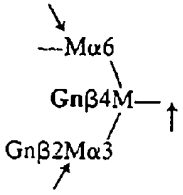
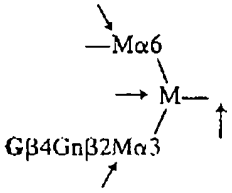
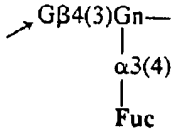
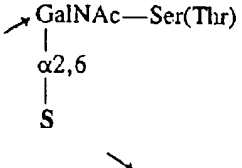
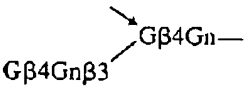
MGn can be acted on by GlcNAc-transferases II, III, or IV, or by α6-fucosyltransferase, or can enter the trans Golgi. The GlcNAc-transferase II catalyzed conversion of MGn to GnGn is probably the "main-line" pathway (step 8, Fig. 3). GnGn is the main entry point into all complex *N*-glycosyl oligosaccharides. GnGn can be acted on by (*i*) GlcNAc-transferase III to form bisected biantennary complex structures, (*ii*) either GlcNAc-transferase IV or V to form nonbisected triantennary complex structures, or (*iii*) α6-fucosyltransferase; or (*iv*) can enter the trans Golgi to be acted on by β4-Gal-transferase (Fig. 5).

The pathway for *O*-glycosyl oligosaccharide synthesis shows similar "crossroads" (Fig. 4). For example, GalNAc-Ser(Thr)-R may be converted to core type 1 (path *b*, Fig. 4), core type 3 (path *c*, Fig. 4), or sialylα2-6GalNAc-Ser(Thr)-R (path *a*, Fig. 4). Core type 1 (Galβ1-3GalNAc-R) may be acted on by several different enzymes (paths *f*–*i*, Fig. 4). Various other crossroads are evident in Figs. 4 and 5.

The existence of these competition points means that many different oligosaccharides can be made in a single assembly line depending on the relative activities of competing enzymes and may explain, at least in part, the microheterogeneity of oligosaccharides at a single amino acid site. In addition, tissues and species probably differ in their complement of competing enzymes, resulting in the differences in structure that have been observed. For example, ovine submaxillary glands have a higher ratio of CMP-sialic acid:GalNAc-R α6-sialyl-

TABLE 3. Substrate specificity: GO – NO GO*



X	a	Ref.
	α-Mase II Gn-T II and IV α6-Fuc-T	45 46 44
	α-Mase II Gn-T II, III, and IV Gn-T V† α6-Fuc-T†	45 29, 46 47 44
	Blood group enzymes: A-dependent α3-GalNAc-T to Gal B-dependent α3-Gal-T to Gal H-dependent α2-Fuc-T to Gal	 48
	β3-Gal-T to GalNAc	49
	Blood group I-dependent β6-Gn-T to Gal	 50

*M, mannose; Gn, *N*-acetylglucosamine; G, galactose; S, sialic acid; Fuc, fucose; Gn-T, *N*-acetylglucosaminyltransferase. The residue in bold print is X, the NO GO signal. The arrows indicate the site of action of the enzymes listed under *a*.

†These enzymes have only been tested with substrates in which there is a Gal residue on both antennae.

transferase (path *a*, Fig. 4) to UDP-Gal:GalNAc-R β3-Gal-transferase (path *b*, Fig. 4) than do porcine submaxillary glands; the α6-sialyl residue prevents β3-Gal-transferase action (path *s*, Fig. 4) and, therefore, ovine submaxillary mucin contains primarily sialylα2-6GalNAc chains whereas porcine submaxillary mucin contains mainly larger chains. The interesting point is that these two mucins differ quantitatively rather than qualitatively in their oligosaccharide compositions. Table 1 lists other tissue and species differences for several of the mucin glycosyltransferases.

Substrate specificity

GO – NO GO

One of the most important directing forces in oligosaccharide biosynthesis are the constraints imposed by the specific substrate requirements of many of the glycosyltransferases. Some of these properties will now be reviewed. The first example has been named the GO – NO GO effect. This simply means that the insertion of a single critical sugar residue (X) into an oligosaccharide will convert it from a substrate to a nonsubstrate. Table 3 lists some of the most important NO GO residues.

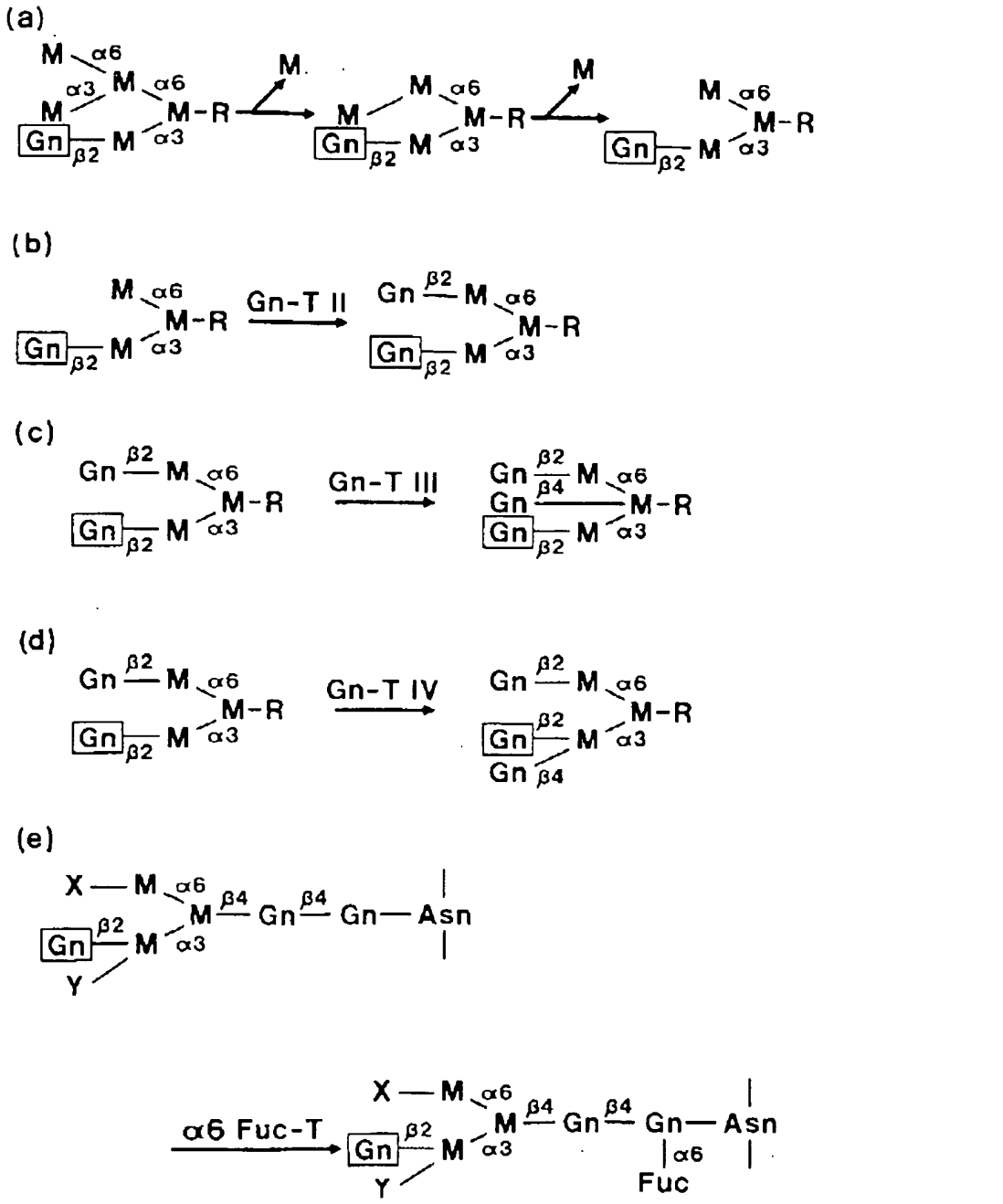


FIG. 7. Reactions catalyzed by five enzymes requiring the prior action of GlcNAc-transferase I which inserts a GlcNAc in β1-2 linkage into the Manα1-3 arm. (*a*) Mannosidase II; (*b*) GlcNAc-transferase II; (*c*) GlcNAc-transferase III; (*d*) GlcNAc-transferase IV; (*e*) α6-fucosyltransferase. M, Man; Gn, GlcNAc; Gn-T, GlcNAc transferase; Fuc, fucose; α6Fuc-T, α6-fucosyltransferase.

The first two examples refer to a group of enzymes acting on *N*-glycosyl oligosaccharides (illustrated in Fig. 7) which have in common the following properties (38, 51): (*i*) they require the presence of the GlcNAcβ1-2Manα1-3Manβ1-4 grouping, (*ii*) activity is blocked if the substrate contains a bisecting GlcNAc (Fig. 7), and (*iii*) activity is blocked if the GlcNAcβ1-2Manα1-3Manβ1-4 antenna is covered at its nonreducing end by a Galβ1-4- residue. We explain these data by assuming that these enzymes all require the GlcNAcβ1-2Manα1-3Manβ1-4 grouping as a recognition site distinct from the catalytic site and that this recognition site is blocked either by a bisecting GlcNAc or by a Gal residue. The structures shown in Figs. 8 and 9 are based on three-dimensional studies by nuclear magnetic resonance spectrometry (34) and illustrate the steric hin-

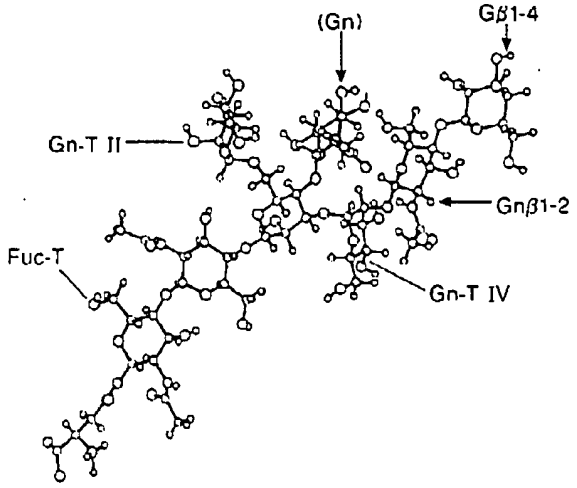


FIG. 8. Computer-drawn structure for MG(Gn), ω = +180°, based on NMR data of Carver and Brisson (34). The sites of action of GlcNAc-transferases II and IV (Gn-T II and IV) and of α6-fucosyltransferase (Fuc-T) are indicated. M, Man; Gn, GlcNAc; (Gn), bisecting GlcNAc; G, Gal.

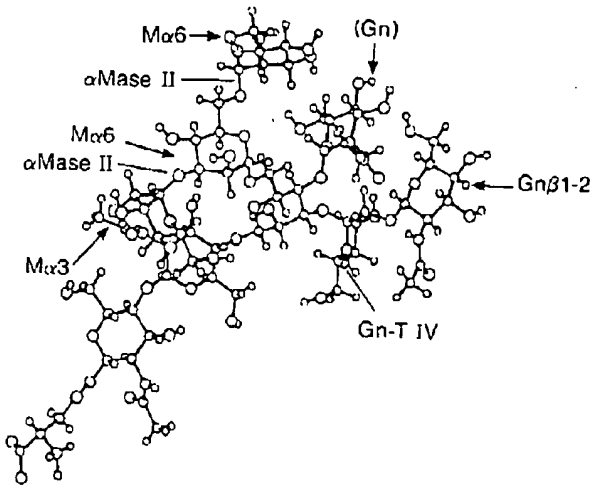


FIG. 9. Computer-drawn structure for Gn(I,III)M_5, based on NMR of Carver and Brisson (34). The sites of action of α-mannosidase II (αMase II) are indicated. Abbreviations are as for Fig. 8.

drance of the recognition site by either the bisecting GlcNAc or Gal residues. Figure 8 shows that the sites of action of GlcNAc-transferases II and IV and of α6-fucosyltransferase are accessible and well removed from the sterically blocked GlcNAcβ1-2Manα1-3Manβ1-4 site; Fig. 9 shows a similar situation for α-mannosidase II.

Other NO GO residues are the following. (*i*) A fucose linked α1-3 or α1-4 to a penultimate GlcNAc residue will prevent the action of blood group A-dependent α3-GalNAc-transferase, blood group B-dependent α3-Gal-transferase, and blood group H-dependent α2-fucosyltransferase. (*ii*) As mentioned in the previous section, a sialyl residue linked α2-6 to GalNAc prevents the action of the mucin core 1 β3-Gal-transferase. (*iii*) A Galβ1-4 residue on the GlcNAcβ1-3Galβ1-4- terminus prevents the action of blood group I β6-GlcNAc-transferase. These examples lend support to the concept of carbohydrate recognition sites remote from the catalytic sites.

NO GO – GO

The NO GO – GO effect is the reverse phenomenon: i.e., the insertion of a single critical sugar residue (Y) into an oligosaccharide will convert it from a nonsubstrate to a substrate. Two typical examples are shown in Table 4. The first example is at an important decision point in the synthesis of *N*-glycosyl oligosaccharides. If GlcNAc-transferase I does not insert a GlcNAc residue on the Manα1-3 arm (step 6, Fig. 3), synthesis of hybrid and complex *N*-glycosyl oligosaccharides cannot occur. This GlcNAc residue is a GO signal for all enzymes shown in Fig. 7 and provides a recognition site for them, as explained in the previous section. The second example in Table 4 has been called the "3-before-6" rule and applies to three enzymatic activities which are probably due to the action of a single β6-GlcNAc-transferase (Ref. 15; I. Brockhausen and H. Schachter, unpublished data). The rule states that the β6-GlcNAc-transferase(s) responsible for synthesis of mucin core 2 (path *d*, Fig. 4) and core 4 (path *e*, Fig. 4) and for the branch point of the blood group I determinant (paths *o* and *p*, Fig. 4) requires substitution of C-3 of the target sugar residue before C-6 can be substituted. Put another way, core 1 synthesis precedes core 2 synthesis, and core 3 synthesis precedes core 4 synthesis.

Since Gal residues appear to be needed for *i* and I antigenic activities, it is not strictly correct to state that blood group *i* synthesis precedes blood group I synthesis. However, the β3-GlcNAc-transferase must act before the β6-GlcNAc-transferase. For example, pig stomach mucosal extracts can transfer GlcNAc in β1-3 linkage to the terminal Gal of Galβ1-3(GlcNAcβ1-6)-GalNAc-R but not to GlcNAcβ1-6Galβ1-3(GlcNAcβ1-6)GalNAc-R (19). Further, we have detected both the GlcNAcβ1-3Gal— and GlcNAcβ1-3(GlcNAcβ1-6)-Gal— structures but not the GlcNAcβ1-6Gal structure as enzyme products (Refs. 19 and 20; I. Brockhausen and H. Schachter, unpublished data).

The above rules, like all the other rules stated in this review, may have exceptions. For example, Yamashita et al. (7) reported the presence of the following structure in hen ovomucoid:

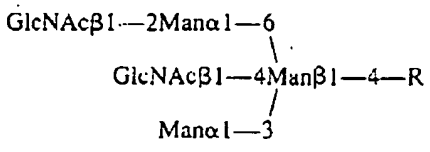
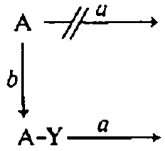
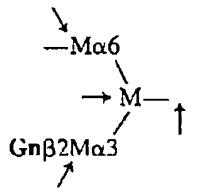
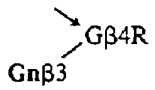


TABLE 4. Substrate specificity: NO GO – GO*



Y	a	Ref.
	α-Mase II	45
	Gn-T II, III, and IV	29, 46
	Gn-T V	47
	α6-Fuc-T	44
Gβ3GalNAc—Ser(Thr)		17
or		18
Gnβ3GalNAc—Ser(Thr)	Mucin core 2 and (or) 4 β6-Gn-T to GalNAc	15, 16
	Blood group I-dependent β6-Gn-T to Gal	50, 52,†

*M, Man; Gn, GlcNAc; G, Gal; Gn-T, GlcNAc-transferase. The residue in bold print is Y, the GO signal. The arrows indicate the site of action of the enzymes listed under *a*.

†I. Brockhausen, A. Koenderman, K. L. Matta, D. H. Van den Eijnden, and H. Schachter, in preparation.

This structure does not fit the synthetic rules because it implies that both GlcNAc-transferases II and III have acted without the presence of the GlcNAcβ1-2Manα1-3- grouping. The above compound represented less than 4.6% of the total carbohydrate and may have been formed by glycosidase action.

Another exception to the rules is the report of GlcNAcβ1-6GalNAc in human κ-casein (53). We have been unable to detect the synthesis of this disaccharide *in vitro* in our systems. For example, GalNAc-mucin was incubated with UDP-GlcNAc and enzyme from either pig colon or rat colon, and the product was released by alkaline borohydride and purified by gel filtration and high performance liquid chromatography; analysis by high resolution nuclear magnetic resonance spectrometry and methylation analysis revealed GlcNAcβ1-3GalNAcOH to be the only disaccharide product (15). Perhaps human tissues have an unusual β6-GlcNAc-transferase or the residue on C-3 of GalNAc was incorporated and subsequently lost. What is reassuring is that the great majority of structures isolated from many different glycoproteins fit the synthetic rules outlined above.

Recognition sites distinct from catalytic sites

Table 5 gives examples which support the concept of recognition sites distinct from catalytic sites. We have already discussed the first example in which GlcNAcβ1-2Manα1-3Manβ1-4- serves as a recognition site for the enzymes listed in Fig. 7. The second example is the requirement shown by GlcNAc-transferase I for a Manα1-3Manβ1-4GlcNAc- moiety (51). The third example was reported by Joziasse et al. (54) for bovine colostrum CMP-sialic acid:Galβ1-4GlcNAc-R α2-6-sialyltransferase; they noted a partial requirement for an intact GlcNAc ring in the Manβ1-4GlcNAc sequence of the *N*-glycosyl oligosaccharide core and postulated that the sialyltransferase required this region for optimum positioning on the substrate.

Branch specificity

Van den Eijnden and co-workers have coined the term branch specificity to describe the preferential action of a glycosyltransferase on a particular arm of a branched oligosaccharide (Table 6). For example, elongation of biantennary complex *N*-glycosyl oligosaccharides by both β4-Gal-transferase and α2-6-sialyltransferase oc-

TABLE 5. Substrate specificity: recognition sites distinct from catalytic sites

Recognition site	Enzymes	Ref.
R' \ α6 Manβ4GlcNAc—R / α3 GlcNAcβ2Man	α-Mase II	45
	GlcNAc-T II, III, and IV	29, 46
	GlcNAc-T V	47
	α6-Fuc-T	44
R2 \ α6 Manβ4GlcNAc—R / α3 R1—Man	GlcNAc-T I	51
	α2-6-Sialyl-T to Galβ4GlcNAc—	54

TABLE 6. Substrate specificity: branch specificity

Branched oligosaccharide	Enzyme	Preference	Ref.
R1—Mα6 \ Mβ4—R / R2—Mα3	β4-Gal-T	Mα3 arm	55 56
	α6-Sialyl-T	Mα3 arm	57
	α3-Gal-T	Mα6 arm	58
R1—Gnβ6 \ Gal—R / R2—Gnβ3	α3-Gal-T	Gnβ6 arm	58
	β4-Gal-T	Gnβ6 arm	59

curs preferentially on the Manα1-3 arm. This may be due, in part, to the fact that the Manα1-6 arm can exist in two states in equilibrium with each other; one of these states may not be as readily accessible as the other (Ref. 34; Figs. 10 and 11). However, the fact that α3-Gal-transferase from calf thymus prefers the Manα1-6 arm (58) makes this argument somewhat suspect. The indications are that branch specificities found *in vitro* reflect structural variations that occur *in vivo*.

Role of polypeptide

The *in vitro* glycosyltransferase studies described above were carried out either with well-characterized low molecular weight acceptors or relatively poorly characterized glycoprotein acceptors. It is as yet not possible to obtain a pure large polypeptide with a homogeneous oligosaccharide at a single amino acid

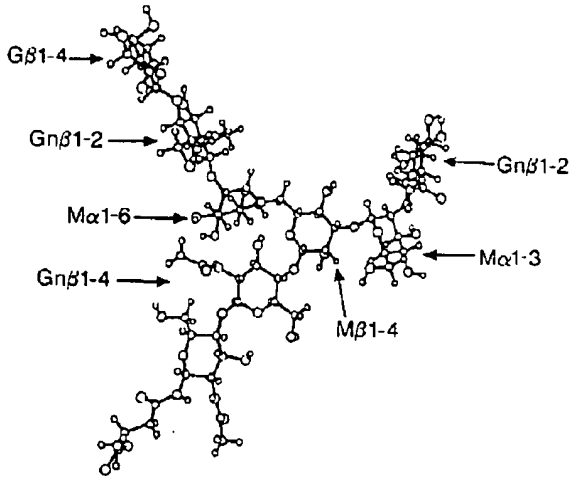


FIG. 10. Computer-drawn structure for GGn, ω = −60°, based on NMR data of Carver and Brisson (34). Abbreviations are as for Fig. 8.

position. Thus, the role of the polypeptide in glycosyltransferase action has not been properly studied. As noted above, however, the rules outlined by *in vitro* transferase studies are usually supported by the structures isolated from tissues. Nevertheless, there are strong indications that the polypeptide backbone often plays an important modifying role during oligosaccharide assembly.

For example, consider the situation for hen ovalbumin and hen ovomucoid (14). Both glycoproteins are made in the same organ and yet ovalbumin contains mainly high mannose and bisected hybrid structures,

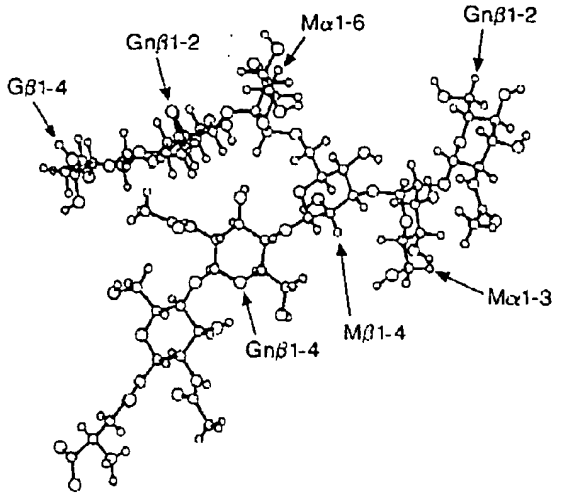


FIG. 11. Computer-drawn structure for GGn, $\omega = +180°$, based on NMR data of Carver and Brisson (34). Abbreviations are as for Fig. 8.

whereas ovomucoid is very rich in bisected highly branched truncated (mainly GlcNAc terminal) complex structures. Both glycoproteins are rich in bisected structures and indeed we have found that hen oviduct is a rich source of GlcNAc-transferase III (29), whereas rat and pig liver lack this enzyme. But why is there a difference between the two proteins? One explanation is that the two proteins pass through separate and different assembly lines. Figure 12 suggests another possible explanation. The polypeptide backbone may interact with oligosaccharide in such a way as to make ovalbumin a good substrate and ovomucoid a poor substrate for GlcNAc-transferase III at the five-mannose stage whereas ovomucoid becomes a good substrate for the transferase at the three-mannose stage.

The reason for the undergalactosylation of ovomucoid and various other glycoproteins is not known. It is interesting that Narasimhan et al. (56) found that not only does a Gal residue on the Manα1-3 arm inhibit GlcNAc-transferase III, but β4-Gal-transferase is inhibited by a bisecting GlcNAc; this is perhaps not surprising on stereochemical grounds (Fig. 8). Thus prior addition of a bisecting GlcNAc may lead to undergalactosylation of the GlcNAcβ1-2Manα1-3- and GlcNAcβ1-2Manα1-6-antennae relative to the other antennae; the structures reported for ovomucoid tend to support this hypothesis (14).

Savvidou et al. (60) have provided evidence for the role of polypeptide–oligosaccharide interaction in the synthetic process. They showed that a human IgG1k monoclonal protein (Hom) had oligosaccharide moieties not only at the usual position (Asn-297 of the H chain) but also at Asn-107 on the L chain. All oligosaccharides were of the complex biantennary *N*-glycosyl type with core fucose residues and, therefore, processing at both sites had entered the Golgi phase (Fig. 3). At this point, the H and L chains are already covalently associated and therefore both glycosylation sites must pass through the same endomembrane assembly line and encounter the same array of processing enzymes. However, the Asn-107 site was occupied entirely by mono- and disialyl bisected biantennary complex structures, whereas the Asn-297 site had mainly (73%) nonbisected sialylated biantennary structures and 27% bisected neutral biantennary structures. Since galactosylation and sialylation occur after the action of GlcNAc-transferase III (Fig. 5), it cannot be argued that the oligosaccharide at Asn-297 is buried in the protein and therefore not accessible to GlcNAc-transferase III. The clone of cells making this IgG was obviously rich in GlcNAc-transferase III, since the Asn-107 site is fully bisected. Why is the Asn-297 site so poorly bisected?

The explanation hinges on the assumption that GlcNAc-transferase III cannot act on the substrate conformation in which the torsional angle ω about the C-5–C-6 bond of the Manα1-6 linkage equals −60° (the two antennae form a Y, Fig. 10). The substrate for GlcNAc-transferase III is the conformation in which the angle ω is +180° (the Manα1-6 arm is bent backwards towards the core, Fig. 11). The basis for this assumption (61) is that, whereas the nonbisected biantennary oligosaccharide exists in an equilibrium between two thermodynamically favourable conformations ($\omega = -60$ or +180°, Figs. 10 and 11), the bisected biantennary structure is found exclusively in the orientation corresponding to $\omega = +180°$ (Fig. 8). It was suggested (61) that the oligosaccharide at Asn-107 exists in equilibrium between both conformations and is therefore accessible to GlcNAc-transferase III, whereas most of the oligosaccharide at Asn-297 is stabilized **by interaction with the polypeptide** in the $\omega = -60°$ conformation and cannot serve as a substrate for GlcNAc-transferase III. The reason for the 27% bisected structures at Asn-297 is not clear. Figure 13 illustrates the hypothesis. In support of this theory, elucidation of the three-dimensional structure of the F_c fragment from a human immunoglobulin, determined by X-ray diffraction studies (62), showed that the nonbisected biantennary complex oligosaccharide at Asn-297 was indeed stabilized in the form with $\omega = -60°$ through interactions between the protein and the terminal Gal residue of the Manα1-6 arm.

Substrate availability and other factors

It has already been pointed out that the endomembrane system is highly compartmentalized (Figs. 3 and 5). The analogy to an assembly line is obvious. The transferases and glycosidases which shape the oligosaccharide structure are arranged along the endomembrane in the order in which they are needed. This arrangement minimizes the need for the substrate to seek its proper

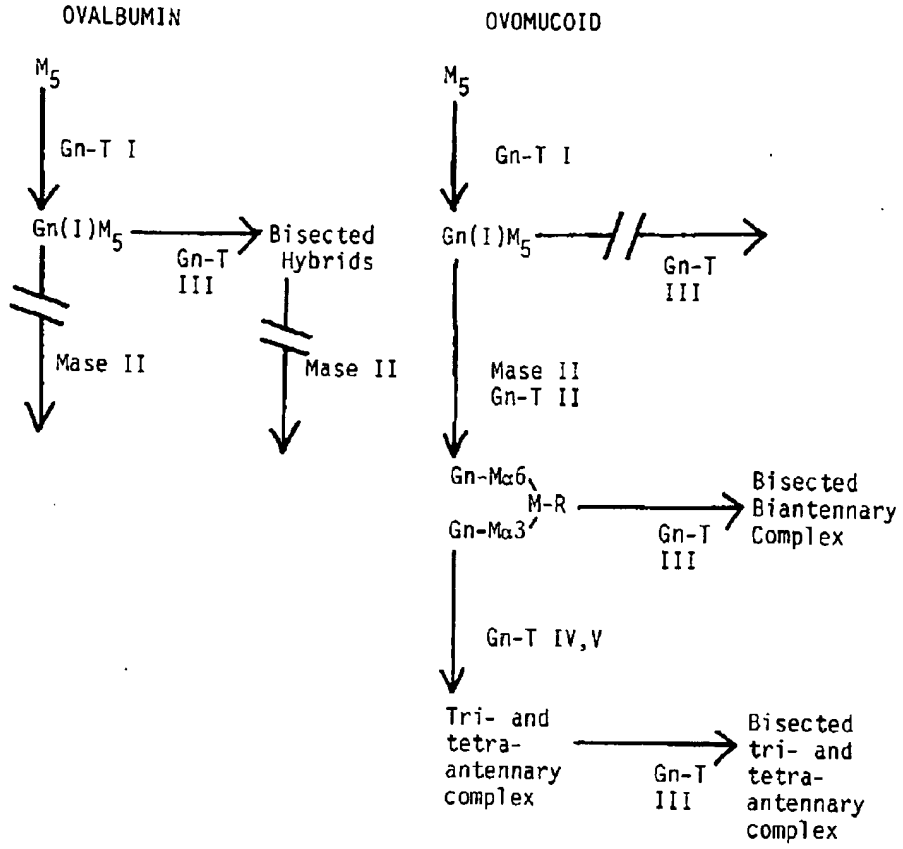


FIG. 12. Suggested mechanism for the difference between ovalbumin and ovomucoid (see text). M, Man: Gn, GlcNAc; Mase II, α-mannosidase II; Gn-T, GlcNAc-transferase.

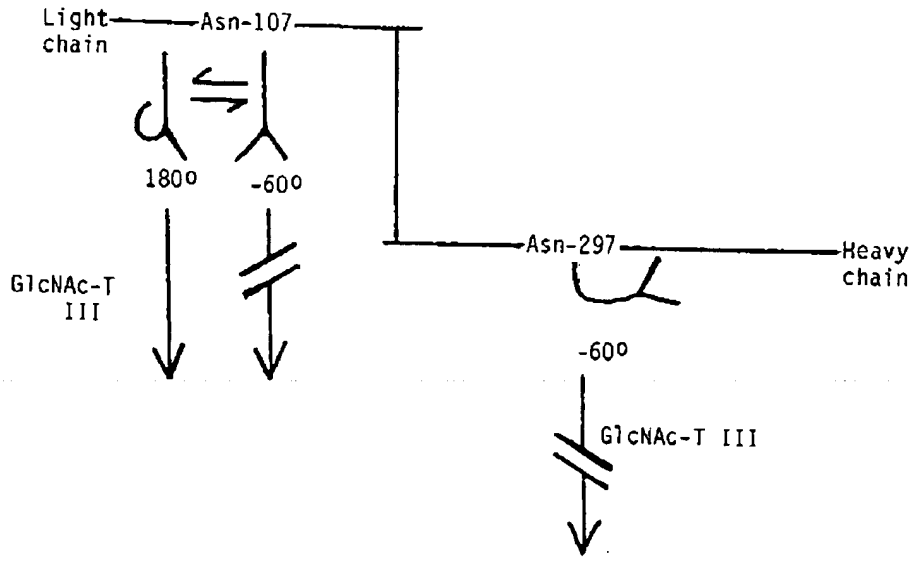


FIG. 13. A monoclonal IgG, studied by Savvidou et al. (60), was found to have bisected oligosaccharides at Asn-107 of the light chains, but mainly nonbisected oligosaccharides at Asn-297 of the heavy chains. Interaction of the oligosaccharide at Asn-297 with the protein backbone is believed to be the basis of this effect (see text).

enzyme and also exercises a certain amount of control over the assembly process.

For example, the fact that the GlcNAc-transferases which control branching (Fig. 6) are in the medial Golgi compartment, whereas the terminal glycosyltransferases (Gal- and sialyl-transferases) are in the trans Golgi (Fig. 5), means that branching is determined before the antennae are completed. This control must be important since the cell uses yet another mechanism to ensure that it is carried out. Once the glycoprotein enters the trans Golgi, it is galactosylated (Fig. 5) and this immediately prevents further action of GlcNAc-transferases II, III, IV, and V and of α6-fucosyltransferase. Thus, even if there are small amounts of these medial Golgi enzymes in the trans Golgi, they are quickly prevented from acting. The reason for this stringent control is not known.

Several laboratories have reported transport proteins within the membranes of the endomembrane system which allow the movement of nucleotide sugars from the cytoplasm into the lumenal spaces. These transport mechanisms are indicated in Fig. 3 and obviously play a major role in controlling glycoprotein synthesis. Defects in these transport proteins have been shown to lead to the disruption of glycoprotein and glycolipid synthesis. However, a discussion of this important topic is beyond the scope of this review.

Finally, a variety of other substrate-level factors may exert control on the synthetic process. (*i*) The pH of the lumenal compartment may affect transferase activity. (*ii*) The availability of cations is usually needed for transferase action. (*iii*) The presence of pyrophosphatases in the Golgi allows conversion of the nucleotide diphosphate formed after glycosyltransferase action into nucleotide monophosphate. The nucleotide monophosphate is exported out of the Golgi apparatus in a counter-transport system with nucleotide sugars. (*iv*) Several laboratories have reported on the importance of phospholipids in glycosyltransferase activity. (*v*) Post-translational modifications of glycosyltransferases, e.g., phosphorylation, may play a role in modifying their activity. These factors will not be discussed in this review.

Conclusions

It is apparent that many different factors enter into the construction of a complex carbohydrate. It is, therefore, not surprising that a large variety of structures is synthesized by the cell. We are beginning to understand some of the biosynthetic machinery involved. However, it has not been ruled out that much of the microheterogeneity of these structures is indeed a random process with little functional significance. The arguments presented in this review indicate that a great deal of control is exerted and raise the hope that as we understand the process better the functional aspects will become more evident.

What is needed are new approaches to the problem. Figure 1 emphasizes the genetic aspects and suggests that one approach must be to obtain genetic probes for the enzyme machinery involved in biosynthesis. Indeed, several laboratories are engaged in this pursuit and a few initial successes have been reported. Perhaps within the next few years, a battery of genetic probes will become available that will allow the fine dissection of the biosynthetic pathways outlined in Figs. 4 and 5.

Another area of future research must be the three-dimensional aspects of protein–oligosaccharide interaction, particularly the role of branching. Such studies are important not only for model systems such as lectin–oligosaccharide interactions (63), but also for the proper understanding of how the biosynthetic machinery operates, of how complex carbohydrates at the cell surface control the interactions of a cell with its environment, and in fact, of all functional aspects of complex carbohydrates.

In conclusion, although tremendous advances have been made in recent years in the structure and biosynthesis of complex carbohydrates, we are only beginning to attack the problem of function.

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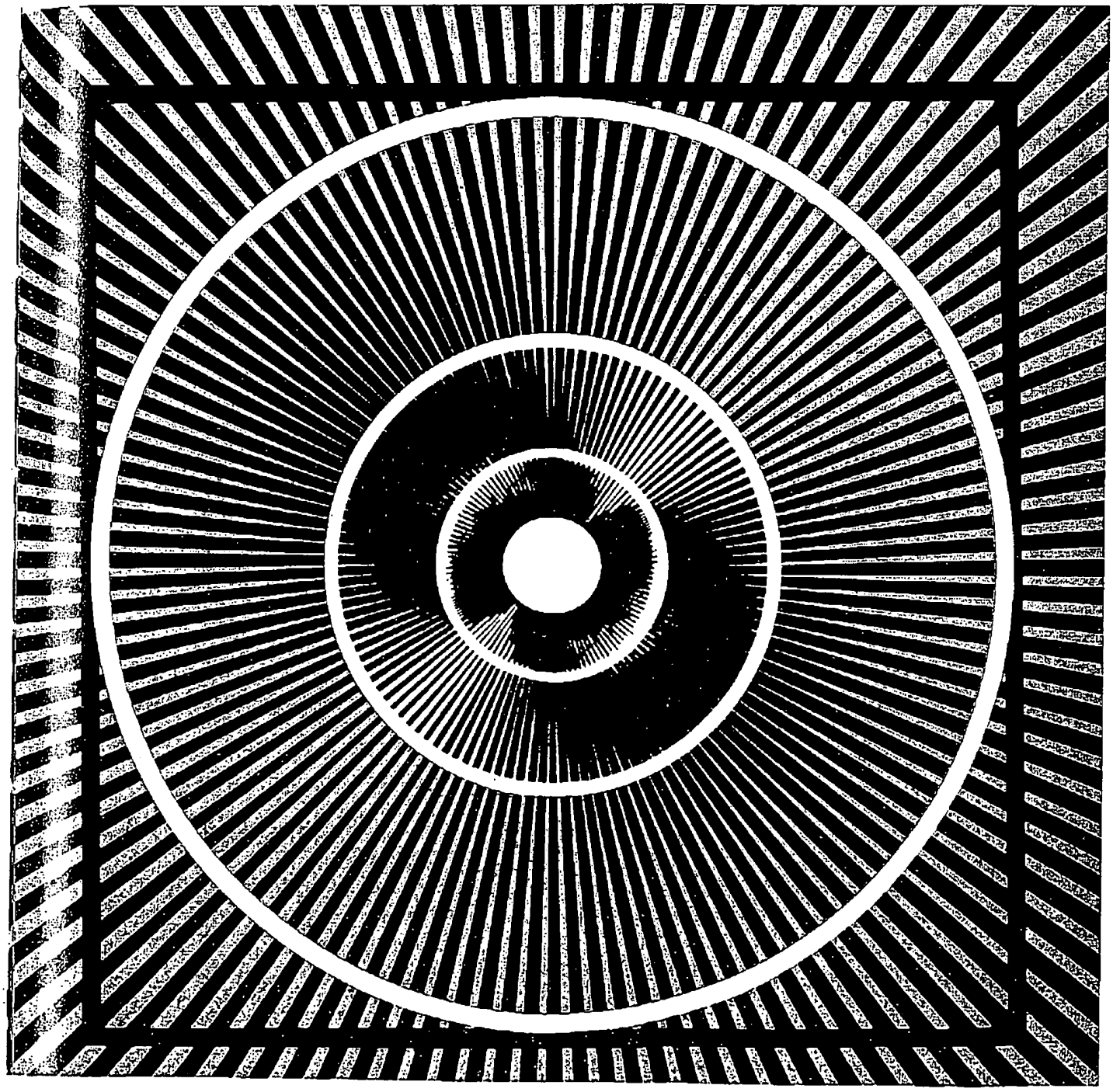

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Glycosylation of Antibody Molecules: Structural and Functional Significance

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The IgG antibody molecule is a structural paradigm for members of the immunoglobulin superfamily. A majority of these molecules are glycoproteins and collectively they account for ~70% of molecules currently undergoing development for possible in vivo therapeutic application. Whilst the oligosaccharide moiety of the IgG molecule accounts for only 2–3% of its mass, it has been shown to be essential for optimal activation of effector mechanisms leading to the clearance and destruction of pathogens. This suggests that glycosylation fidelity is an essential requirement of the IgG molecule and that it may be so for other recombinant glycoproteins produced by in vivo or in vitro techniques. Numerous studies have shown that whilst the defining biological activity of a glycoprotein molecule may not be dependent on its glycosylation, other essential characteristics are altered in aglycosylated forms, e.g. stability, pharmokinetics, antigenicity [1–3].

The IgG molecule is composed of three globular protein moieties, two Fabs and an Fc, that are linked through a flexible 'hinge' region that allows freedom for multiple spatial orientations of the globular moieties with respect to each other. A flexible upper hinge region provides mobility for the Fab regions and allows the paratope of each to bind its complementary epitope. A flexible lower hinge region similarly allows Fc mobility and accessibility within antigen/antibody complexes to engage one of a variety of effector activating ligands, e.g. Fcγ receptors, the C1 component of complement. A core hinge section is rich in proline and cystine residues, that form inter-heavy chain disulphide bridges, and has a rigid secondary structure.

Studies attempting to correlate physicochemical parameters with function were interpreted to suggest that the segmental flexibility of the hinge region

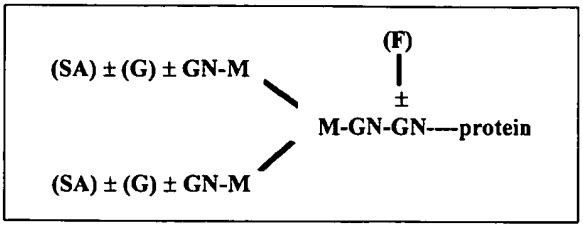

Fig. 1. The core carbohydrate moiety of the complex form of oligosaccharides is represented by the sugar residues in open type. The possible outer arm residues are bracketed. All possible combinations are observed. SA = Sialic acid; G = galactose; GN = N-acetylglucosamine; M = mannose; F = fucose. N-linked attachment of oligosaccharide occurs on the amide side chain of the Asn-x-Ser/Thr sequon (x ≠ Pro); the Ser/Thr residue forms hydrogen bond(s) with the amide group in order to activate it for attachment to the primary N-acetylglucosamine residue of the dolichol intermediate, by oligosaccharyltransferase.

was directly related to the ability of an IgG molecule to activate complement; rather than indirectly by allowing access to interaction sites in the C_H2 and/or C_H3 domains for effector ligands [4]. The validity of these conclusions has recently been re-evaluated by the application of protein engineering techniques in an attempt to introduce rational structural changes predicted to affect biological activity. The results of these studies demonstrate the necessity for Fc glycosylation and that protein/oligosaccharide interactions determine the generation of a structure that is permissive of Fc-ligand recognition and activation, while failing to confirm a primary role for the hinge region. Our studies suggest that whilst the oligosaccharide moiety may not contribute directly to ligand binding, except for mannan-binding protein, it does exert a subtle influence on protein tertiary and quaternary structure that is essential to 'wild type' activity. Consequently, Fc-ligand recognition, and hence biological activity, may be modulated by judicious replacement of amino acid residues that contribute to non-covalent protein/oligosaccharide interactions.

Antibody Glycosylation

Human antibody molecules of the IgG class have N-linked oligosaccharide attached at the amide side chain of Asn297 on the β-4 bend of the inner (Fx) face of the C_H2 domain of the Fc region [5]. The oligosaccharide moiety is of the complex biantennary type having a hexasaccharide 'core' structure (GlcNAc2Man3GlcNAc) and variable outer arm 'non-core' sugar residues, such as fucose, bisecting N-acetylglucosamine, galactose and sialic acid (fig. 1).

Thus, a total of 36 structurally unique oligosaccharide chains may be attached at each Asn297 residue. It is anticipated that glycosylation can be asymmetric so that an individual IgG molecule may have different oligosaccharide chains attached at each of the Asn297 residues within the Fc region such that whilst the heavy chain synthesised within a single antibody-secreting cell may be homogeneous in its amino acid sequence glycosylation can result in the production of $(36 \times 36)/2 = 648$ structurally unique IgG molecules or glycoforms; NB: the total number of combinations is divided by two because of the two-fold symmetry of the molecule. Analysis of monoclonal and polyclonal IgG demonstrates the presence of all the predicted oligosaccharide species, however, disialylated oligosaccharides may be absent or present at a very low level [6]. With the additional possibility of the presence of complex N-linked oligosaccharide in the Fab region, it is apparent that glycosylation is a post-translational modification that can introduce a very significant structural and, possibly, functional heterogeneity into the IgG molecule. The presence of additional glycosylation sites within the heavy chains of the other Ig isotypes means that the possible number of glycoforms may be orders of magnitude higher [7, 8].

The Fc glycosylation site is a conserved feature for all mammalian IgGs investigated and glycosylation occurs at a homologous position in human IgM, IgD and IgE, molecules, but not in IgA. Human IgM, IgA, IgE and IgD molecules bear additional N-linked oligosaccharide moieties attached to the constant domains of the heavy chains and IgA subclass 1 (IgA1) and IgD proteins also bear multiple 0-linked sugars in their extended hinge regions, attached to hydroxyl groups of serine and threonine residues. It has been estimated that ~30% of polyclonal IgG molecules also bear an oligosaccharide moiety within the Fab region. Since the sequences of the constant region of κ and λ light chains and the C_H1 domain of the heavy chain do not include a glycosylation sequon, the oligosaccharide of glycosylated Fab regions is due to attachment within either the V_L or V_H sequences. Analysis of the DNA sequences of 83 human germline V_H gene segments revealed five that encoded potential glycosylation sites, however, none of these sequons were observed in 37 V_H protein sequences – detailed analysis to determine whether the germline gene from which these proteins were derived did encode a glycosylation sequon was not attempted. Fifteen of the 37 protein sequences did have potential glycosylation sequons which, it would appear, have resulted from somatic mutation and antigen selection [7, 8]. It has been demonstrated that the structure and function of Fab oligosaccharide can depend on the site of attachment. Thus, monoclonal murine anti-dextran antibodies with a single oligosaccharide attachment site at residues 54, 58 or 60 in complementarity-determining region 2 (CDR2) were shown to have differing antigen-binding activities [9]. A monoclonal human polyreactive autoantibody, secreted by a heterohybridoma cell

line was shown to be glycosylated on both the V_L and V_H regions; the V_H glycosylation site was at residue 75 in framework 3, adjacent to CDR3 [10].

Structural Consequences of IgG Glycosylation

Whilst glycosylation of the IgG/Fc is essential for optimal expression of effector activities mediated through FcγR and the C1 component of complement direct interaction of the oligosaccharide moiety with these effector ligands has not been demonstrated. Recently, it was reported, however, that in agalactosylated IgG the oligosaccharide moiety 'flips' out of the inter-C_H2 space and the terminal N-acetylglucosamine residues become available to bind and activate mannan-binding protein [11], and consequently the classical complement cascade. By contrast, these residues are not available to the lectin *Bandeireae simplicifolia* II in the native form of agalactosylated IgG but become so on denaturation. Resolution of structure for the oligosaccharide chains in x-ray crystallography demonstrates that it is not freely mobile and has definite conformation. From its attachment point at Asn297, it 'runs forward' towards the C_H2/C_H3 interface region and it is estimated that 82 non-covalent interactions between core sugar residues and the outer arm residues of the $\alpha[1\rightarrow6]$ arm may be possible [12]. Together with the sugars of the $\alpha[1\rightarrow3]$ arm, the oligosaccharide fills the available volume between the C_H2 domains. It may be anticipated, therefore, that whilst interactions with the Fx face of the protein impose structure on the oligosaccharide chain there is a reciprocal influence of the oligosaccharide on the protein structure.

The structural and functional consequences of Fc glycosylation can be assessed by comparison of glycosylated and aglycosylated forms of IgG. The latter can be generated by production in *Escherichia coli*, growing IgG producing cells in the presence of the glycosylation inhibitor tunicamycin or by protein engineering of the glycosylation sequon. It should be appreciated that IgG produced in *E. coli* or in the presence of tunicamycin will have an asparagine residue at 297 whilst site-directed mutagenesis can introduce any chosen amino acid residue; in the present case, alanine. A more subtle approach is to isolate homogeneous glycoforms for structural and functional studies or to replace individual or combinations of amino acid residues that make contacts with sugar residues. The latter approach may allow a detailed understanding of the oligosaccharide/protein interactions in this molecule, the 'rules' of template direction and its effect on the type of oligosaccharide attached and the generation of mutant molecules with new profiles of biologic function.

A small, localised protein structural change has been detected for aglycosylated human chimeric IgG3 and its Fc fragment by ^{1}H-NMR. Previous studies

had allowed assignments for each of the five histidine residues, and their distribution through the Fc makes them suitable probes for detection of localised structural change. Such a change was reported for His268 which is in the vicinity of both the carbohydrate attachment site and the lower hinge binding site on IgG for Fc receptors [13]. A similar spectral difference was observed between a glycosylated IgG1 Fc fragment and the aglycosylated form produced as a recombinant protein in *E. coli* [14]. A structural difference between the lower hinge regions (residues 234–237) of glycosylated and aglycosylated IgG was inferred from the different papain cleavage profiles obtained for glycosylated and aglycosylated mouse IgG2b. Whilst a single cleavage point at residue 229 was observed for the glycosylated protein, the aglycosylated mutant was cleaved heterogeneously at residues 228, 234, and 235 [15].

Recent ^{13}C-NMR studies have provided direct evidence of differing structural dynamics for the lower hinge residues of glycosylated and aglycosylated mouse IgG2b [Kato, Lund and Jefferis, unpubl.]. Significant differences are revealed when thermodynamic parameters are determined from data obtained from differential scanning microcalorimetry of glycosylated and aglycosylated mouse IgG2b-Fc [Tischenko, Lund and Jefferis, unpubl.]. Differences are observed in both the C_H2 and C_H3 domains and the free energy of stabilisation of the C_H2 domain is decreased. An attempt to monitor structural differences between glycosylated and aglycosylated human IgG3 through altered epitope expression, employing a panel of > 30 mouse monoclonal anti-human Fcγ antibodies, did not detect any loss of expression or obvious reduction in affinity.

Functional Consequences of Asn297 Glycosylation

Since it has been consistently demonstrated that glycosylation is essential for optimal expression of FcγR- and C1-mediated effector functions, it may be anticipated that biological activity may vary between differing glycoforms. Most studies have compared differences between natural forms of IgG and their aglycosylated or agalactosylated counterparts; however, we have added the approach of generating mutant proteins in which residues reasoned to participate in oligosaccharide-protein interactions have been replaced.

A wide range of effector cells are activated by IgG/antigen immune complexes through interactions with cellular receptors for the Fc region of the gamma chain, FcγR. Three types of human Fc receptors (FcγRI, FcγRII, and FcγRIII) have been defined, by gene cloning and sequencing, that are differentially expressed on a variety of cell types; additionally FcγR may be induced or their expression up-regulated following cellular activation. The IgG isotype specificity of the FcγR suggests that recognition is correlated with

primary amino acid sequence. An earlier prediction that the lower hinge residues 234–237 (-Leu-Leu-Gly-Gly-) in particular might correlate with FcγRI recognition appeared to be confirmed by protein engineering studies with the demonstration that replacement of any one of these residues in mouse/human chimeric IgG3 affected recognition by all three human FcγR [16, 17]. It was proposed, therefore, that the three Fc receptors are recognised by overlapping, non-identical ligand-binding sites. This appears rational for a family of receptors that are evolutionarily related and exhibit a high degree of sequence homology.

Other structural features are also determinants for recognition since replacement of Pro331 by serine, the amino acid residue present at this position in IgG4, reduces the binding affinity for IgG1 and IgG3 by an order of magnitude [18]; these residues are within 11 Å of the lower hinge. The FcγRII receptor is polymorphic and the allelic forms are designated as FcγRIIa-H131 and FcγRIIa-R131 to indicate that a histidine/arginine interchange at residue 131 is critical to recognition of human IgG2 molecules [19]. Thus, monocytes of homozygous H/H131 individuals were found to internalise IgG2-opsonized erythrocytes more efficiently than cells from R/R131 individuals. Since the lower hinge region of IgG2 molecules is radically different from that of IgG1 and IgG3, it is apparent that the recognition site for FcγRII depends on structure outside this region.

The amino acid replacement studies suggest that FcγR recognition is dependent on a precise molecular architecture and that subtle structural changes have a dramatic effect on biological function. This conclusion is further supported by the demonstration that aglycosylated human chimeric IgG3 has a reduced interaction with all three Fc receptors [16, 17, 20]. Whilst hapten-derivatised red blood cells could still be sensitised with this antibody to trigger superoxide production by U937 cells, stimulated with γ-interferon, higher levels of sensitisation were required compared to glycosylated IgG3 [20]. The aglycosylated IgG3 was not recognised by human FcγRII expressed on K562 and Daudi cells [21] and rosette formation mediated through FcγRIII, expressed on human NK (natural killer) cells, was reduced to 40% of that obtained for glycosylated IgG3, whereas antibody-dependent cellular cytotoxicity (ADCC) was essentially abolished [17]. Comparative studies of a glycosylated and an aglycosylated humanised anti-CD3 antibody suggest that the altered biological activities of aglycosylated IgG may be exploited for some in vivo applications. In the model investigated the glycosylated IgG was able to effect immune modulation and was immunogenic; probably due to its ability to activate T cells following interactions with appropriate FcγR-expressing cells. By contrast, the aglycosylated antibody was not immunomodulatory, was less immunogenic and had a longer half-life [22].

Attempts to evaluate the contribution of outer arm sugars to biologic function have concentrated mostly on glycoforms differing in galactose content. In a sustained investigation of the EBV-transformed lymphocytes secreting anti-D antibody, it has been shown that antibody with a high galactose content (>70% digalactosyl IgG) was more effective than antibody with a low galactose content (10% agalactosyl and 50% monogalactosyl IgG) in FcγRI- and FcγRIII-mediated cellular lysis (ADCC) [23]. An evaluation of the contribution of galactosylation to FcγRI recognition was made by comparison of the ability of a low galactose (<20% galactosylated) and a fully galactosylated form of an IgG4 Fc to inhibit superoxide generation through mouse/human chimeric IgG3, no difference was detectable in this system [24]. A minimal reduction in FcγR and a 2-fold reduction in C1q binding for agalactosyl IgG relative to the galactosylated form has been reported [25].

Considerable clinical experience has been gained with the humanised monoclonal antibody Campath-1H and its promise requires optimisation of control and efficiency of production. The product of rat YO, chinese hamster ovary (CHO) and mouse NSO cells has been evaluated for glycosylation and ADCC activity [26]. Interestingly, the rat cells were demonstrated to secrete IgG with relatively high levels of bisecting GlcNAc and to be the most active of the three products in ADCC, leading to the conclusion that this glycoform may have significant biologic advantage. The product of the NSO cells was reported to be underglycosylated. The final conclusion was that the cell type was a more important parameter than the culture conditions, at least for medium with and without added serum. It should be noted, however, that the method of culture used for each cell type was significantly different; the YO cells were grown in roller bottles, the NSO cells in shaking flasks and the CHO cells in hollow fibre bioreactors. In our experience [27], these differences in the method of culture could account, in large part, for the differences in glycoform profiles observed. The influence of outer arm sugars was evaluated for Campath-1H antibody following exposure to neuraminidase and β-galactosidase; removal of low levels of sialic acid had no effect on ADCC or complement-mediated lysis (CML), however, whilst removal of galactose was without effect on ADCC, it resulted in ~50% reduction in CML activity [28].

The essential requirement for protein/core-oligosaccharide interactions with a biantennary-type oligosaccharide is suggested from studies of a chimeric mouse-human IgG1 antibody produced in Lec-1 cells which are incapable of processing high mannose forms of oligosaccharide [29]. The antibody product having a high mannose oligosaccharide attached at Asn297 was incapable of complement-mediated hemolysis and deficient in C1q and FcγRI binding. In contrast a chimeric mouse-human IgG1 antibody produced in yeast cells, with presumed incorporation of high mannose forms of oligosaccharides at Asn297,

maintained the ability to trigger ADCC through human FcγRIII [30]. The importance of C_H2 domain protein/core-oligosaccharide interactions in IgG is emphasised by the demonstration that recognition by Fcγ receptors can be modulated in mutant proteins in which core oligosaccharide contact residues have been replaced. Thus, replacement of Asp265, a contact residue for the primary GlcNAc residue of the core oligosaccharide, resulted in reduced recognition by human FcγRI and human FcγRII. By contrast, replacement of non-core contact residues Lys246, Asp249 by Ala and Glu258 by Asn was without effect on recognition for these receptors, a finding consistent with the view that the interactions with GlcNAc and Gal residues of the Manα(1→6) arm are not essential for maintenance of recognition by human FcγRI and FcγRII [24].

The biological half-life of a recombinant glycoprotein is a vital property determining in vivo efficacy and the economics of treatment. Studies of blood clearance of glycosylated and aglycosylated mouse/human chimeric IgG1 in mice demonstrated accelerated clearance for the aglycosylated form but with similar half-lives. Since the half-life of IgG1 in humans is ~23 days but measured as 5 days in this model it is difficult to draw a definitive conclusion. Catabolism of aglycosylated mouse IgG2b was evaluated in a rat model and shown to be cleared more rapidly than the glycosylated form and it was concluded that the increased catabolism occurred in the extravascular space [31]. The plasma half-lives and bioavailability of human anti-D antibodies secreted by Epstein-Barr virus (EBV)-transformed human B cells, cultured in hollow-fibre bio-reactors, have been evaluated in vivo in comparison with polyclonal anti-D isolated from immunised volunteers [32]. The half-lives of an IgG1 and an IgG3 monoclonal anti-D antibody were 22.2 and 10.2 days, respectively, compared to 15.6 days for polyclonal anti-D IgG. The half-life of polyclonal anti-D IgG was dependent on the proportions of IgG1 and IgG3 present in the preparation. Studies of mutant mouse IgG1 proteins have been interpreted to localise the site controlling catabolism to the inter C_H2/C_H3 region and to demonstrate modulation of the half-life [33].

Evaluation of a panel of 28 mutant mouse IgG2b proteins, each with a surface accessible amino acid replacement, for C1q binding and C1 activation correlated recognition with the presence of the wild-type residues lysine, glutamic acid and glutamic acid at 318, 320 and 322 [34]. A contrary result has been reported for mouse/human chimeric IgG1 antibody with the demonstration that replacement of glutamic acid 320 was without effect on CML, however, C1 activation was abrogated following amino acid replacements in the lower hinge region [35]. This is consistent with the observation that a Pro→Ser replacement at residue 331 in IgG1 and IgG3 results in a reduced capacity to trigger complement lysis [36]. One of the mutant proteins produced by Duncan

and Winter [34] was Asn→Ala, 297 which results in the production of aglycosylated mouse IgG2b. This protein had a 3-fold reduced capacity to bind human C1q and a much reduced ability to trigger lysis of target cells with guinea pig complement through the classical complement cascade. Similarly, an aglycosylated mouse/human chimeric IgG1 was shown to retain some ability to trigger lysis of target cells by human complement but with a 7- to 8-fold higher antibody concentration requirement than for the glycosylated wild type IgG1 [37]. These data suggest a similarity in the molecular requirements for FcγR and C1 recognition and that glycosylation is essential for generation of a quaternary structure expressing these ligand binding sites.

The role of outer arm sugars in C1 mediated lysis has been investigated for galactosylated and agalactosylated IgG, produced following exposure to β-galactosidase, with an observed 2-fold higher activity for the galactosylated form [25]. Confirmation of the importance of correct glycosylation is provided by study of a human-mouse chimeric IgG1 molecule produced in yeast cells and anticipated to have high mannose type oligosaccharide attached at Asn297 [29]. The IgG1 product was unable to activate C1 to trigger human complement mediated lysis of targets whilst the same chimeric IgG1 construct expressed in rodent cells (Sp2/0) was effective. A direct role for the oligosaccharide moiety in activating the complement cascade is apparent for the lectin mannan-binding protein which can function as a surrogate C1 component. The specificity of mannan-binding protein is for mannose and N-acetylglucosamine residues, and it has been shown that it can access and bind to terminal N-acetylglucosamine residues exposed on agalactosyl IgG [11].

Much interest has been generated by the observation of a deficit in IgG galactosylation in patients with rheumatoid arthritis (RA) and some other inflammatory diseases, including tuberculosis and Crohn's disease [38]. Another feature of RA is the presence in the blood of rheumatoid factor (RF) autoantibodies having specificity for epitopes in the Fc region of IgG. Since RFs are, typically, of IgM or IgG isotype, the immune complexes formed have the potential to trigger effector functions through IgG-mediated pathways or a combination of IgG and IgM pathways. The chronic inflammatory reactions resulting are thought to contribute erosive damage in this disease. A dominant specificity of RFs is for an epitope localised to the area of contact and interaction between the C_H2 and C_H3 domains. This specificity overlaps with that of Staphylococcal protein A and the binding of a majority of RFs to IgG can be inhibited by Staphylococcal protein A [8, 39]. It has been speculated that terminal galactose or sialic acid residues on the α[1→6] arm of the oligosaccharide may be accessible to RFs and influence recognition and binding affinity, with a consequent effect on the nature and size of immune complex formed. A galactose residue on the α[1→6] arm is resolved on x-ray crystallography

and possible non-covalent contacts identified. It has been argued, therefore, that this galactose residue occupies a lectin-like pocket that will be exposed in agalactosylated IgG and may contribute to altered IgG antigenicity, e.g. reactivity with RFs [38]. Alternatively, the mannose-binding protein provides a route by which agalactosylated IgG could trigger the inflammatory reactions seen in RA independently of RFs [11].

A study of the reactivity of 16 monoclonal RFs generated from synovial tissue lymphocytes with IgG of differing galactose content (18–86%) yielded ambiguous results. Five RFs reacted more avidly to IgG of low galactose content, 6 were not influenced by galactose content and one bound more avidly to IgG of high galactose content [40]. A comparison of the binding of polyclonal and monoclonal RFs to glycosylated and aglycosylated chimeric mouse/human IgG proteins of each of the subclasses detected no differences for IgG1, IgG2 and IgG4 proteins, however, RFs reactive with IgG3 proteins reacted more avidly with aglycosylated IgG3 [41]. In a companion study, some monoclonal RFs were found to bind aglycosylated IgG4 less well than glycosylated IgG4 (2- to 5-fold), suggesting that the carbohydrate moiety is important in establishing their binding epitope in the C_H2 domain [42]. An interesting difference between the latter two studies was that for one the source of monoclonal RF was serum of patients with Waldenströms macroglobulinemia [41] and for the other EBV-transformed synovial tissue lymphocytes of RA patients. Given the parallelism between RF and Staphylococcal protein A binding to IgG, it is pertinent to note that there is only one report of a minimal effect of glycosylation on the binding of Staphylococcal A to IgG. An interesting recent study demonstrated isotype regulation mediated through the generation of auto-anti-isotype antibodies (RFs) during the course of an immune response to influenza virus. A series of RFs were established as monoclonal antibodies and demonstrated to effect immune deviation in vivo. One of these RFs, a monoclonal IgA RF, specific for mouse IgG2b bound the aglycosylated protein poorly [43].

On complexing with polyvalent antigen, IgM is able to initiate the classical complement cascade, following binding of C1q molecule to the C_H3 domain; the equivalent of the C_H2 domain of IgG. Amino acid replacements within glycosylation sequon 402–404 [44] of the C_H3 domain of mouse IgM results in a 3- to 25-fold decrease in the capacity to effect CML of target cells by guinea pig complement. This lowered activity could be due, at least in part, to an observed 4- to 8-fold reduction in assembly of the monomeric subunits into pentameric and hexameric IgM molecules. Replacement of residue 406 (Ser→Asn), analogous to core contact residue 301 in IgG, resulted in a 50-fold decrease in the capacity of mouse IgM to trigger lysis through guinea pig complement [45]. These data suggest that interactions between amino acid

residues and core sugar residues of the oligosaccharide attached at Asn-402 of the IgM molecule may be important for the formation of the C1-binding and activation site.

Factors Influencing Glycosylation Hybridoma, and Recombinant Immunoglobulin Molecules

Regulatory authorities demand exhaustive testing of monoclonal antibodies that might be applied for in vivo diagnostic or therapeutic purposes. If approved, a similar demand for the demonstration of product consistency is made. The parameters that should be analysed, in vitro, include isotype, subclass, affinity, microheterogeneity, molecular weight, primary and secondary structure, structural integrity, specificity, glycosylation profile, biological potency. Subsequently, the product would be evaluated for pharmacological, toxicological, biodistribution and half-life in vivo [3]. Functional studies of recombinant human proteins have established that the form of the oligosaccharide moiety attached at a specific glycosylation site should be the same as that attached to the natural molecule. Regulatory authorities require authentic and consistent glycosylation of molecules that may be applied in vivo, therefore, animal cells are preferred to other systems for their production. The biotechnology industry has concentrated on development of production protocols employing CHO cells for all recombinant human glycoproteins demonstrating that its glycosylation machinery is catholic and that the polypeptide chain has a major influence on the type of oligosaccharide attached. However, CHO cells do not satisfy industrial economic requirements for the production of antibodies and so there has been a resurgence of interest in NSO cells that are derived from an antibody secreting plasmacytoma. A recent study also employed the rat plasmacytoma line YO and demonstrated its product to have a natural glycosylation profile that included the presence of bisecting GlcNAc residues [26]. For any given cell type, glycosylation of antibody products remains a variable dependent on numerous parameters that include, the method of cell culture, the supply of nutrients, removal of metabolic products, when the protein is harvested and a subtle influence of the polypeptide chain on outer arm sugar heterogeneity. A further concern is the possibility that mutant clones may arise during extensive and continuous culture with the emergence and overgrowth of a sub-clone secreting structurally and functionally aberrant molecules. The reality of this concern is demonstrated by the isolation of multiple sub-clones of CHO cells each of which expresses an altered profile of glycosyltransferases and consequently secretes glycoproteins with unique glycoform profiles [46].

Experience in the production of mouse/human chimeric antibodies in J558L cells demonstrated significant differences in galactosylation depending on whether it was produced in shallow culture, hollow fibre bioreactor or in vivo, as ascitic fluid. Of particular concern is the production of variable proportions of molecules bearing additional galactose residues in α[1→3] linkage to normal galactose sugars. This results from the activity of an endogenous α[1→3]-galactosyltransferase. Gene expression for this enzyme is depressed in humans and higher primates with the result that it constitutes an immunogenic structure and it has been estimated that 1% of circulating human IgG is 'anti-Gal' antibody [47] and its presence can be readily demonstrated in an ELISA [48]. Although the CHO cell line expresses an α[1→3] galactosyltransferase, there appears to be only one documented instance in which it has been demonstrated to be active in the addition of Gal α[1→3] Gal [49]. We have had the experience of culturing multiple clones of transfected J558L whose antibody product had an essentially normal glycoform profile over several years; then, for reasons unknown to us, the antibody product included high mannose oligosaccharides and low site occupancy [Lund, Takahashi and Jefferis, unpubl. obs.]. A similar experience has been reported for human IgG1 and IgG2 antibodies produced by heterohybridomas for which variable proportions of high mannose containing antibody was obtained [50]. Glycosylation appeared to be dictated by the mouse plasmacytoma partner since N-glycolylneuraminic acid but no bisecting GlcNAc was added. Similarly, a humanised anti-CD18 antibody produced in NSO cells was shown to contain five oligomannoside-type structures in addition to the usual biantennary-type oligosaccharide moieties, no bisecting GlcNAc and no sialic acid [51]. The glycosylation status of human anti-D antibody produced by EBV-transformed lymphocytes grown at low density in static culture or high density in hollow fibre bioreactors also demonstrated high levels of galactosylation for antibody produced at low density and a relatively natural profile of glycoforms for antibody produced in the bioreactor [23]. Heterohybridomas secreting anti-D antibody have also been established; however, analysis showed that 12/16 such cell lines had incorporated Galα[1→3]Gal epitopes into the antibody [52].

A further rodent/human difference is in the form of sialic acid utilised. Polyclonal human IgG has a terminal N-acetyl neuraminic acid sugar on ~25% of oligosaccharides, by contrast the mouse utilises N-glycolyl neuraminic acid. Interestingly, chimeric mouse/human IgG3 produced in J558L cells was shown to be a mixture of molecules having one or the other derivative. This demonstrates that both transferases are available and that their utilisation is affected by subtle structural effects. A bisecting GlcNAc residue is present in ~10–20% human polyclonal IgG but NSO and CHO cells lack the GlcNAc transferase III enzyme required for its addition. The extensive functional

studies reported for recombinant molecules produced in CHO cells suggested that bisecting GlcNAc has little influence on biological activity, however, the presence of glycoforms with bisecting GlcNAc produced by rat YO cells has been held to account for its beneficial biological activity [26]. These findings point to the need for a productive cell line of human origin, however, none is available that has a high endogenous rate of protein synthesis. Many other vehicles for recombinant protein production are being appraised or under development. The early promise of *E. coli* has not been realised for glycoproteins since bacteria do not have a glycosylation machinery [14]. Experiences with insect cells differ. Thus whilst a mixture of high mannose and complex N-linked oligosaccharides was reported for recombinant human plasminogen, including a fully elongated biantennary form (28%) [53] the conclusion drawn from a study of the N-glycosylation of a virion protein was that insect cells were not capable of elongation with the addition of galactose and sialic acid [54]. It is evident that this system is very sensitive to culture conditions and the timing of infection with baculovirus. It is unlikely that other expression systems, such as transfected potatoes, tomatoes, are likely to allow production of glycoproteins that will be acceptable for therapeutic use; it should be remembered that glycosylation is only one of several post-translational events that are essential to the synthesis of a natural form of proteins and glycoproteins.

Whilst one might attempt to develop optimal growth conditions for basic scientific studies, it is likely that they would be too costly to translate into commercial production protocols. Ideally, one would aim for a system that mimics in vivo conditions (homeostasis!) as closely as possible with the maintenance of nutrient concentrations, oxygen tension, removal of metabolites. An unknown factor is the presence of essential growth factors (cytokines) in vivo. A major consideration for biotechnology companies is the overall cost of production and an important element in its determination is downstream processing. Isolation and purification is simplified by the use of defined media and there has been a sustained development of serum-free media with most companies adopting their own undisclosed formulation. Large-scale production facilities have employed air-lift fermenters of 10–12,000 litres capacity. There is a gradual scale-up with the growth of a 'charge' for the next fermenter to allow exponential growth. At the end stage, the cells exhaust the medium, die and protein is released following rupture of the cell wall.

Hollow-fibre bio-reactors have been used for research and intermediate scale production of glycoproteins, including antibodies to be used as in vivo therapeutics. This system does allow continuous exchange between the medium that the cells are suspended in and the 'external' circulating medium. However, the cells are not homogeneously dispersed throughout the cell compartment

but grow in clumps of solid tissue with the result that mass transfer across such a tissue is inefficient and necrosis follows.

We have commented on the heterogeneity of glycosylation of IgG produced in vivo by healthy human adults and instanced altered galactosylation patterns in certain inflammatory diseases. It remains to be determined whether the IgG producing plasma cells are or are not abnormal per se or are developing and producing IgG in an abnormal environment. A fundamental question is whether this is a disease-specific phenomenon that has direct implications for cause and progression or an epiphenomenon that may be used to monitor disease activity and may have value as a prognostic indicator, as for α1-acid glycoprotein [55]. Analysis of mouse and human monoclonal IgGs has demonstrated that each clone exhibits a unique Fc glycosylation profile and, therefore, that the profile for polyclonal IgG is the sum of the many contributing clones. The human monoclonal IgG proteins have been isolated from sera of patients with the disease multiple myeloma. Analysis of a panel of IgG paraproteins with multiple examples of each subclass revealed a subtle template direction effect such that the apparent preference, in polyclonal IgG, for galactosylation of the α[1→6] arm over the α[1→3] arm was reversed for IgG2 proteins and for 2/3 IgG3 proteins [6]. In an extension of these studies, we have observed the α[1→3] preference for a further five IgG3 paraproteins whilst the polyclonal IgG isolated from the same serum sample demonstrated the opposite preference [56]. A further observation is hypogalactosylation of both the polyclonal and the monoclonal IgG, relative to normal polyclonal IgG; however, this did not correlate with IL-6 levels in the same serum sample. It has been shown that IL-6 is a growth factor for plasma cells proliferating in the bone marrow in this disease and that it is reflected in increased IL-6 levels in the serum [57].

For proteins with multiple glycosylation sites, fidelity is observed for the type of oligosaccharide attached at each. Such template direction, excluding outer arm sugars, is exemplified for IgM, IgA, IgE and IgD molecules. Thus, for a mouse IgM secreting plasmacytoma, the oligosaccharide attached at Asn171 is a biantennary complex form, oligosaccharides at Asn332, 364 and 402 are triantennary and at Asn563 predominantly chitobiose ($Man_3GlcNac_2$) [58]. Similarly, in the human IgD molecule an oligomannose form is attached at residue 354 within the C_H2 domain, and complex forms at residues 445 and 496 within the C_H3 domain [59, 60]. While large-scale steric effects have been invoked in influencing accessibility of glycosylation sites to glycosylation enzymes, it is less widely appreciated that smaller-scale changes can also affect glycan synthesis. Repositioning of a carbohydrate attachment site within the Fab region of an antidextran antibody by two residues from Asn58 to Asn60 [9], resulted in the attachment of oligomannose forms in place of a complex form and was accompanied by ≥3-fold reduction in affinity for antigen. This

study employed mouse hybridoma cells for antibody production and noted that the Fab oligosaccharide was more fully processed than the Fc moiety and that Galα[1$\rightarrow$3]Gal was added to a significant proportion of Fab oligosaccharide. A more subtle influence of glycosylation status has been demonstrated for a human hybridoma that has a glycosylated λ-chain; variations in glucose availability were shown to determine the size of the oligosaccharide attached and the antigen-binding activity [61]. Alternatively, it has been shown that glycosylation sequons can be introduced into variable regions with consequent glycosylation that does not affect antigen binding and which can be used for conjugation of haptens [62].

The extensive interactions between the oligosaccharide and protein moieties suggest the possibility to modulate them by selected amino acid replacements with a consequent influence on biological activity. Such an effect has been observed on replacement of the core contact residue Asp265 by Ala (DA265), resulting in greatly increased levels of galactosylation but a diminution of FcγRI-mediated function; 50% more oligosaccharide chains with galactose residues on both the α[1$\rightarrow$3] and α[1$\rightarrow$6] arms for mouse/human chimeric IgG3 produced in CHO cells [63].

In conclusion, it is evident that post-translational glycosylation of proteins can have subtle and more far-reaching structural and functional consequences. These consequences will be particularly manifest for recombinant glycoproteins produced in vitro but intended for in vivo application. A second rapidly developing area of interest results from the observation of altered glycosylation states for specific proteins correlating with disease and/or disease activity. The field is set to expand as sensitive technologies for determining oligosaccharide structures and profiles are now commercially available.

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Modulation of Therapeutic Antibody Effector Functions by Glycosylation Engineering: Influence of Golgi Enzyme Localization Domain and Co-Expression of Heterologous β1, 4-*N*-acetylglucosaminyltransferase III and Golgi α-mannosidase II

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Abstract: The effector functions elicited by IgG antibodies strongly depend on the carbohydrate moiety linked to the Fc region of the protein. Therefore several approaches have been developed to rationally manipulate these glycans and improve the biological functions of the antibody. Overexpression of recombinant β1,4-*N*-acetylglucosaminyltransferase III (GnT-III) in production cell lines leads to antibodies enriched in bisected oligosaccharides. Moreover, GnT-III overexpression leads to increases in non-fucosylated and hybrid oligosaccharides. Such antibody glycovariants have increased antibody-dependent cellular cytotoxicity (ADCC). To explore a further variable besides overexpression of GnT-III, we exchanged the localization domain of GnT-III with that of other Golgi-resident enzymes. Our results indicate that chimeric GnT-III can compete even more efficiently against the endogenous core α1,6-fucosyltransferase (α1,6-FucT) and Golgi α-mannosidase II (ManII) leading to higher proportions of bisected non-fucosylated hybrid glycans ("Glyco-1" antibody). The co-expression of GnT-III and ManII led to a similar degree of non-fucosylation as that obtained for Glyco-1, but the majority of the oligosaccharides linked to this antibody ("Glyco-2") are of the complex type. These glycovariants feature strongly increased ADCC activity compared to the unmodified antibody, while Glyco-1 (hybrid-rich) features reduced complement-dependent cytotoxicity (CDC) compared to Glyco-2 or unmodified antibody. We show that apart from GnT-III overexpression, engineering of GnT-III localization is a versatile tool to modulate the biological activities of antibodies relevant for their therapeutic application.

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Keywords: glycosylation engineering; antibody-dependent cellular cytotoxicity (ADCC); complement-dependent cytotoxicity (CDC); therapeutic IgG; chimeric GnT-III

INTRODUCTION

Antibodies of the IgG class have proven to be useful anticancer therapeutics (Carter, 2001). Their high specificity for an antigen, for targeting a cancerous cell, and the simultaneous recruitment of immune effector cells, by binding to Fcγ receptors (FcγRs) via their Fc region, make them a powerful tool for immunotherapies. This linker function of the antibodies results in the elimination of the cancerous cell by cell-mediated effector functions, such as antibody-dependent cellular cytotoxicity (ADCC).

Many therapeutic antibodies depend on Fc-mediated effector functions and it was concluded that they require a higher in vivo efficacy to increase their potential as therapeutic drugs. The recruitment of FcγR-expressing cells relies on an efficient binding to the Fc region of IgG (Dall'Ozzo et al., 2004). The affinity of this interaction can be improved by amino acid mutations of the polypeptide (Shields et al., 2001), which bear the risk of immunogenicity. On the other hand the presence of specific oligosaccharide structures linked to the Cγ2 domain of the Fc fragment was reported to affect the biological activity of the antibody (Jefferis et al., 1998; Lifely et al., 1995; Wright and Morrison, 1997) by influencing the interaction with FcγRs (Tao and Morrison, 1989). In this context, modification of the carbohydrate moiety associated to the Fc region of IgG has proven to be a successful approach to

enhance ADCC (Shields et al., 2002; Shinkawa et al., 2003; Umaña et al., 1999).

Recombinant DNA-based glyco-engineering for increased antibody effector function was first achieved by over-expression of heterologous β1,4-*N*-acetylglucosaminyltransferase III (GnT-III), in antibody-producing cells (Umaña et al., 1999). GnT-III catalyzes the addition of a bisecting *N*-acetylglucosamine (GlcNAc) to N-linked oligosaccharides, as long as they have been modified by *N*-acetylglucosaminyltransferase I (GnT-I) and have not been modified by β1, 4 galactosyltransferase (GalT). Therefore, any non-galactosylated hybrid or complex oligosaccharide, whether fucosylated or not, can be modified by GnT-III. However, once GnT-III adds a bisecting GlcNAc to an oligosaccharide, other central reactions of the biosynthetic pathway such as core-fucosylation and conversion of hybrid to complex glycans are blocked (Schachter, 1986). This gives GnT-III a high degree of control over the glycosylation process in the Golgi apparatus. Overexpression of GnTIII in antibody-producing cells results in the formation of bisected, non-fucosylated oligosaccharides linked to the antibodies that mediate increased ADCC (Shields et al., 2002; Shinkawa et al., 2003; Umaña et al., 1999).

Previously, we have shown that the GnT-III expression level has a large impact on the relative levels of complex and hybrid, fucosylated, or non-fucosylated oligosaccharides (Umaña et al., 1999). Besides the expression level, the Golgi localization domain of GnT-III, which controls its spatial distribution relative to other enzymes, is another variable influencing the impact of GnT-III on the glycosylation pathway (Nilsson et al., 1996). Here we explore the localization variable by fusing the catalytic domain of GnT-III to the localization domain (cytoplasmic, transmembrane, and stem region) of other Golgi-resident enzymes of the *N*-glycosylation pathway. The resulting chimeric proteins were expressed in antibody-producing cells to engineer the antibody glycosylation pattern and the associated antibody effector functions.

MATERIALS AND METHODS

Construction of Expression Vectors

The DNA for the variable heavy (VH) and variable light (VL) chain of the anti-CD20 antibody was assembled by polymerase chain reaction (PCR) on the basis of the published sequence of the murine C2B8 antibody (Kobayashi et al., 1997; Reff et al., 1994). The IgG1 constant regions were amplified from a human leukocyte cDNA library (BD Biosciences, Allschwil, Switzerland). The rat *GnT-III* gene was amplified using specific primers from a rat kidney cDNA library (BD Biosciences) and a sequence coding for a C-terminal c-myc-epitope tag was added. The construction of the GnT-III-chimeric genes was performed by subsequent overlapping PCR reactions. The DNA fragments coding for the localization domains (cytoplasmic, transmembrane, and stem regions) of human GnT-I (102 amino acids), ManII (100 amino acids), GnT-II (103 amino acids), and α1, 6-fucosyltransferase (α1,6-FucT) (101 amino acids) were amplified from different material of human origin using the specific primers. The gene coding for Golgi α-mannosidase II was amplified by PCR from human DNA using specific primers. The gene coding for the human *N*-acetylglucosaminyltransferase II (GnT-II) was amplified from pGnTII (RG002551, Invitrogen AG, Basel, Switzerland) by PCR using specific primers. The construction of the catalytically inactive GnT-III^{ManII} (iGnT-III^{ManII}) was performed as described (Ihara et al., 2002). The construction of two mutant GnT-III^{mutManII} was accomplished by site-directed PCR mutagenesis, where the mutations R60Q, R73N, L79S, and E81S (GnT-III$^{mutManII(4aa)}$) or R73N, L79S, and E81S (GnT-III$^{mutManII(3aa)}$) were introduced into ManII (Nilsson et al., 1996). All expression vectors were combined with an origin of replication from the Epstein Barr virus (oriP) for episomal vector replication and maintenance in cells producing the Epstein Barr virus nuclear antigen (EBNA). Expression of the protein was confirmed by Western blot detection of GnT-III C-terminal c-myc tag.

For the generation of the FcγRIIIa-expressing CHO cell line, an expression vector for FcγRIIIa-Val158 α-chain, γ-chain, and the gene conferring puromycin resistance was constructed. The cDNAs coding for the FcγRIIIA and the γ-chain were amplified from a healthy donor using specific primers. Genotyping for the FcγRIIIA-Val/Phe158 and FcγRIIC polymorphisms were performed as described (Koene et al., 1997; Metes et al., 2001).

Production and Purification of Glyco-Engineered Anti-CD20 Antibodies in HEK293-EBNA Cells

HEK293-EBNA cells, a kind gift from Rene Fischer (Laboratory of Organic Chemistry, ETH Zürich, Switzerland), were grown as adherent monolayer cultures using DMEM culture medium supplemented with 10% FCS (Invitrogen AG) and were transfected essentially as described by Jordan et al. (1996). HEK293-EBNA cells were used as a transient expression system where the episomal replication of the expression vectors allowed high antibody titres and high expression levels of the glycosylation enzymes. Glyco-engineered antibodies were produced by co-transfection of the cells with two plasmids coding for antibody and chimeric GnT-III, at a ratio of 4:1, respectively, while for unmodified antibody the plasmids coding for the carbohydrate-modifying enzymes were omitted. For the combination of the chimeric GnT-III^{ManII} and ManII, cells were co-transfected with three expression vectors coding for antibody, GnT-III^{ManII} and ManII at a ratio of 3:1:1. The same ratio was used for the combination of GnT-III and ManII, and for that of ManIIGnT-III and GnT-II. At day 5 post-transfection, supernatant was harvested and monoclonal antibody purified using two sequential chromatographic steps as described (Umaña et al., 1999), followed by size exclusion chromatography (HiLoad™ 16/60 Superdex™ 200 column, Amersham Biosciences, Otelfingen, Switzerland).

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Oligosaccharide Analysis

Oligosaccharides were enzymatically released from the antibodies by *N*-Glycosidase digestion (PNGaseF, EC 3.5.1.52, QA-Bio, San Mateo, CA) at 0.05 mU/μg protein in 2 mM Tris, pH7 for 3 h at 37 °C. A fraction of the PNGaseF-treated sample was subsequently digested with Endoglycosidase H (EndoH, EC 3.2.1.96, Roche, Basel, Switzerland) at 0.8 mU/μg protein and incubated for 3 h at 37 °C. The released oligosaccharides were incubated in mild acid (150 mM acetic acid) prior to purification through a cation exchange resin (AG50W-X8 resin, hydrogen form, 100–200 mesh, BioRad, Reinach, Switzerland) packed into a micro-bio-spin chromatography column (BioRad) as described (Papac et al., 1998). The oligosaccharide samples were then analyzed with sDHB as matrix (Papac et al., 1998) using an Autoflex MALDI/TOF (Bruker Daltonics, Faellanden, Switzerland) in positive ion mode. For the assignment of an oligosaccharide structure to each peak, Endoglycosidase H was used due to its specificity. It digests most hybrid and high mannose, but not complex oligosaccharides. A refined oligosaccharide analysis was necessary to distinguish between bisected hybrid and complex (*m/z* 1,339 and 1,502) and their fucosylated versions (*m/z* 1,648 and 1,810), as both structures are not digested by EndoH. For this purpose, EndoH analysis was combined with in vitro galactosylation of the whole antibody performed as described (Raju et al., 2001). The aim was to distinguish between hybrid or complex oligosaccharides by making use of the fact that hybrid oligosaccharides can be galactosylated only at one terminal GlcNAc residue.

Binding of Monomeric IgG1 Glycovariants to Natural Killer (NK) Cells and FcγRIIIa-Expressing CHO Cell Line

Human NK cells were isolated from freshly isolated peripheral blood mononuclear cells (PBMC) applying a negative selection enriching for CD16- and CD56-positive cells (MACS system, Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). The purity determined by CD56 expression was between 88% and 95%. Freshly isolated NK cells were incubated in PBS without calcium and magnesium ions (3×10^5 cells/mL) for 20 min at 37 °C to remove NK cell-associated IgG. Cells were incubated at 10^6 cells/mL at different concentrations of anti-CD20 antibody (0, 0.1, 0.3, 1, 3, 10 μg/mL) in PBS, 0.1% BSA. After two washes with PBS, 0.1% BSA antibody, binding was detected by incubating with 1:200 FITC-conjugated $F(ab')_2$ goat anti-human, $F(ab')_2$ specific IgG (Jackson ImmunoReasearch, West Grove, PA), and anti-human CD56-PE (BD Biosciences, Shields et al., 2002). The anti-FcγRIIIa 3G8 $F(ab')_2$ fragments (Ancell, Bayport, MN) were added at a concentration of 10 μg/mL to compete binding of antibody glycovariants (3 μg/mL). Fluorescence intensity was determined for CD56-positive cells on a FACSCalibur (BD Biosciences) and refers to the geometric mean measured for different antibody concentrations, from which the geometric mean of cells incubated without primary antibody was subtracted.

CHO cells were transfected by electroporation (280 V, 950 μF, 0.4 cm) with an expression vector coding for the FcγRIIIa-Val158 α-chain and the γ-chain. Transfectants were selected by addition of 6 μg/mL puromycin and stable clones were analyzed by FACS using 10 μL FITC-conjugated anti-FcγRIII 3G8 monoclonal antibody (BD Biosciences) for 10^6 cells. Binding of IgG1 to FcγRIIIa-Val158-expressing CHO cells was performed analogously to the NK cell binding described above by omitting CD56-staining.

Biological Activity of Anti-CD20 Monoclonal Antibody Glycovariants

Antibody-Dependent Cellular Cytotoxicity (ADCC) Assay

CD20-positive Raji cells (DMEM, 10% FCS, 1% Glutamax, Invitrogen AG) were labeled with the fluorescent dye Calcein AM for 20 min, according to the manufacturer's instruction (Molecular Probes, Leiden, The Netherlands). Antibodies were serially diluted in AIM-V (Invitrogen AG) and incubated with the target cells for 10 min at room temperature prior to the addition of effector cells. PBMCs were prepared from a donor heterozygous for FcγRIIIa-Val/Phe158 and lacking FcγRIIc expression using Histopaque-1077 (Sigma-Aldrich, Buchs, Switzerland) following the manufacturer's instructions. PBMCs were added to the wells at an effector to target ratio of 25:1. After 4 h incubation at 37 °C, the cells were spun down, washed twice with PBS without calcium and magnesium ions, and lysed by addition of 50 mM borate, 0.1% Triton X-100 solution. The content of the wells was subsequently transferred to a 96-well black flat-bottomed plate. Retention of the fluorescent dye by intact target cells was measured with a fluorometer (485 nm excitation, 520 nm emission, FLUOstar Optima, BMG Labtechnologies, Inc., Durham, NC). Specific lysis was calculated relative to the total lysis control, resulting from incubating the target cells with 1% Triton X-100. Percentage of specific antibody-mediated cytotoxicity was calculated as follows:

$$\%\ \text{cytotoxicity} = ((\text{Fluorescence at concentration } x - \text{Fluorescence of spontaneous release}) / (\text{Fluorescence maximal release} - \text{Fluorescence spontaneous release})) \times 100$$

Each antibody dilution was analyzed in quadruplicate.

Complement-Dependent Cytotoxicity (CDC) Assay

CD20 positive human B lymphoblastoid SKW 6.4 cells (DMEM, 10% FCS, 1% Glutamax, Invitrogen AG) were incubated with increasing concentrations of antibody for 10 min at room temperature prior to the addition of normal human serum (NHS, FcγRIIIA-Val158 and FcγRIIC homozygous donor), prepared from the blood of healthy

volunteers, as source of complement. The blood was allowed to coagulate for 1 h at room temperature and then centrifuged at 1,200*g* for 20 min. The serum was diluted threefold with AIM-V and added to the wells to obtain a final concentration of 20% NHS. The assay plates were incubated for 2 h at 37°C. Maximal release was determined by incubating the cells in the presence of 1% Triton X-100. Relative cytotoxicity was determined using the AlamarBlue™ Assay (Serotec, Inc., Düsseldorf, Germany). Fluorescence was monitored on a fluorometer (540 nm excitation, 590 nm emission, FLUOstar Optima, BMG Labtechnologies, Inc.). Each antibody concentration was measured in quadruplicate.

Whole Blood B Cell Depletion Assay

Four hundred ninety microliters heparinized blood from a healthy FcγRIIIA-Val/Phe158 and FcγRIIC negative donor, were incubated at 37°C with 10 µL PBS or 50-fold concentrated antibody glycovariants (final concentrations of 0.1, 1, 10, 100, 1,000 ng/mL). After 24 h 50 µL blood were stained with a mixture of anti-CD19-PE, anti-CD3-FITC, and anti-CD45-CyChrome (BD Biosciences) for 15 min at room temperature. Before analysis, 500 µL PBS containing 2% FCS and 5 mM EDTA were added to the tubes. The CD3-FITC and CD19-PE fluorescence of the blood samples were flowcytometrically analyzed by gating on all CD45-positive cells. B cell-depletion was determined by plotting the ratio of CD19-positive B cells to CD3-positive T cells. Each antibody concentration was analyzed in triplicate.

RESULTS

Generation of Anti-CD20 Antibody Glycovariants Achieved by Co-Expression of GnT-III or Chimeric GnT-III Proteins

Modulation of antibody glycosylation was achieved by co-expression of the genes coding for antibody and chimeric GnT-III proteins in HEK293-EBNA cells. In the chimeric proteins, the localization domain of GnT-III was replaced with those of various other Golgi-resident enzymes of the N-linked glycosylation pathway (Fig. 1A). The oligosaccharide profiles obtained for the expressed antibodies indicate a significant impact of the enzyme localization on the outcome of the glycosylation process (Fig. 2A, Table I). The expression of GnT-III leads to antibodies with high proportions of bisected oligosaccharides, which can be fucosylated or non-fucosylated and of the complex or hybrid type. The assignment of these structures to the peaks was confirmed by digestion with EndoH, an enzyme digesting high mannose and most hybrid, but not complex oligosaccharides. Thus, peaks at *m/z* 1,664 and 1,810 shift to 1,460, and those at 1,826 and 1,972 to 1,622, confirming the increase in bisected, non-fucosylated hybrid (*m/z* 1,664 and 1,826) and their fucosylated versions (*m/z* 1,810 and 1,972) upon recombinant expression of GnT-III (Fig. 2A and B).The use of the localization domain of GnT-I,

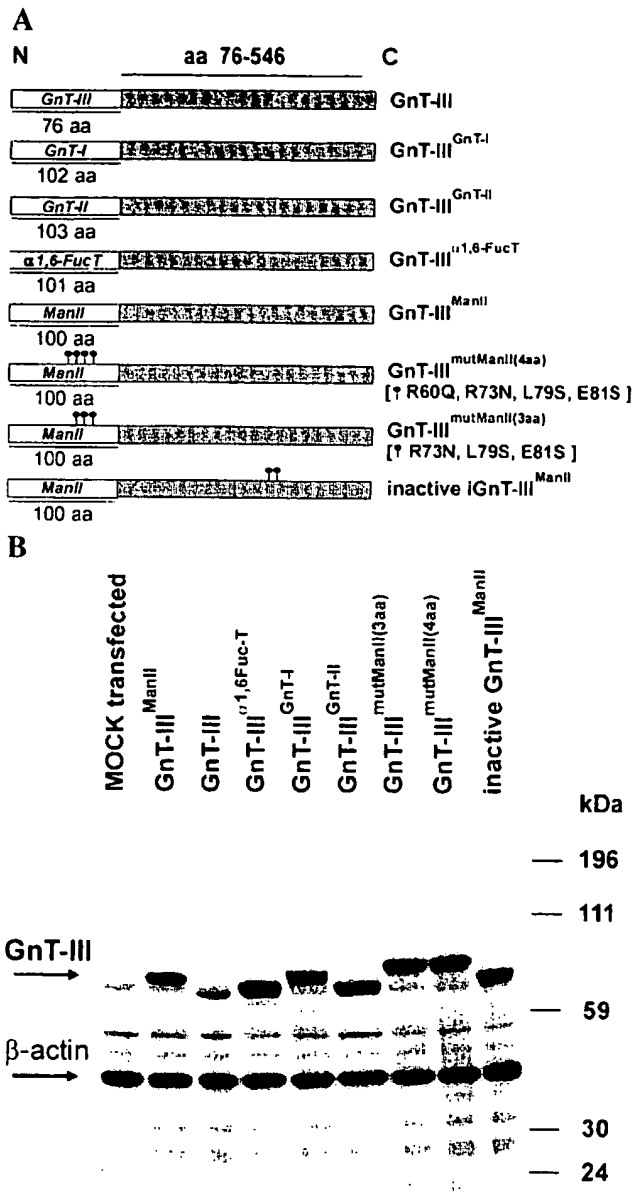

Figure 1. A: β1,4-*N*-acetylglucosaminyltransferase III (GnT-III) chimeric proteins. GnT-III with different localization domains were constructed by fusing the catalytic domain of GnT-III (gray) to the localization domain CTS (white), consisting of cytoplasmic tail, transmembrane domain, and stem region of other Golgi-resident enzymes. The length of the respective regions is indicated. In the last three constructs single amino acid substitutions are indicated. B: Western blot detection of GnT-III. Fifteen micrograms of cell lysates were separated on a 4–12% NU-PAGE gel (Invitrogen AG, Basel, Switzerland) and electroblotted to nitrocellulose membrane. Detection of GnT-III was performed via its C-terminal c-myc tag, while β-actin detection served as an internal control for the amount of loaded extracts (Abcam Ltd, Cambridge, UK).

α1,6-FucT, GnT-II, or ManII, instead of that of GnT-III results in a further increase in the proportion of bisected non-fucosylated hybrid oligosaccharides (mainly *m/z* 1,664) linked to the secreted antibody. Among these chimeric proteins, GnT-III fused to the localization domain of ManII (GnT-III^{ManII}) leads to the highest content of these carbohydrates (Table I, Fig. 2A).

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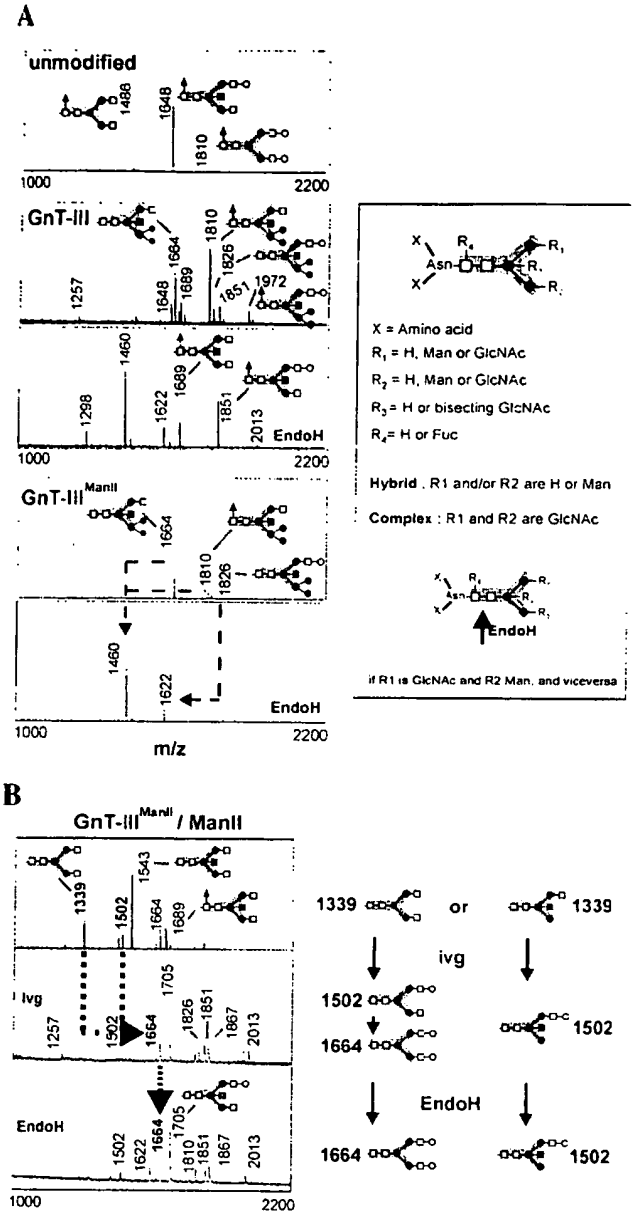

Figure 2. MALDI-TOF-MS spectra of neutral oligosaccharides from recombinant anti-CD20 IgG1 glycovariants. The antibodies were produced in HEK293-EBNA cells engineered for expression of various forms of GnT-III (see Fig. 1a). The *m/z* value corresponds to the sodium-associated oligosaccharide ion. A: MALDI-TOF-MS spectra of neutral oligosaccharides released from unmodified, GnT-III-modified, and GnT-III^{ManII}-modified antibodies. Endoglycosidase H digestion (EndoH) was performed to confirm the increase in bisected non-fucosylated hybrid (*m/z* 1,664 and 1,826) and their fucosylated versions (*m/z* 1,810 and 1,972) upon expression of recombinant GnT-III. EndoH digestion of the released oligosaccharides causes a shift for peaks at *m/z* 1,664 and 1,810 (to *m/z* 1,460) and of peaks at *m/z* 1,826 and 1,972 (to *m/z* 1,622). Complex oligosaccharides, as peaks at *m/z* 1,689 and 1,851 are not digested by EndoH. B: MALDI-TOF-MS spectra of neutral oligosaccharides released from antibodies engineered by co-expression with GnT-III^{ManII} and ManII genes. In vitro galactosylation ("ivg") was performed to refine the oligosaccharide analysis and confirm that the peak *m/z* 1,339 corresponds to complex non-fucosylated oligosaccharides.

Table 1. Oligosaccharide distributions (relative percentages) of anti-CD20 monoclonal antibody glycoforms.

	Unmodified	GnT-III	GnT-III^{GnT-I}	GnT-III^{GnT-II}	GnT-III$^{2,6-FucT}$	GnT-III^{ManII}	GnT-III$^{mutManII(Asn)}$	GnT-III$^{mutManII-dual}$	iGnT-III^{ManII}	GnT-III^{ManII}/ManII	GnT-III/ManII
High mannose	1	2	21	6	3	—	3	3	1	—	1
Hybrid											
Non-bisected fucosylated	v3	—	—	—	—	—	...	—	6	—	—
Non-bisected non-fucosylated	—	—	—	...	—	—	—	—	—	—	—
Bisected fucosylated	...	29	15	35	27	10	38	33	—	1	—
Bisected non-fucosylated	—	22	64	52	66	90	56	62	2	18	3
Complex											
Non-bisected fucosylated	95	5	—	6	4	—	3	2	83	8	8
Non-bisected non-fucosylated	1	—	—	...	—	—	—	—	8	15	13
Bisected fucosylated	—	42	—	1	—	—	—	—	—	15	26
Bisected non-fucosylated	—	—	—	—	—	—	—	—	—	43	49
Total bisected	—	93	79	87	93	100	94	95	2	77	78
Total non-fucosylated	—	22	64	52	66	90	56	62	10	76	65
Total non-fucosylated, complex	1	—	—	—	—	—	—	—	8	58	62
Total complex	96	47	—	7	4	—	3	2	91	81	96

Designation according to Figure 1a.
Unmodified antibody is produced by transient expression in HEK293-EBNA cells by omitting the expression vectors coding for the carbohydrate-modifying enzymes.
The percentages obtained are relative to the total sum of the intensity of each peak.
For hybrid bisected oligosaccharides, additional EndoH digestion confirmed their assignment.

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To assess if the localization domain of ManII itself and not the enzymatic activity of the catalytic domain of GnT-III accounts for any of the oligosaccharide modifications, a catalytically inactive iGnT-III^{ManII} chimeric protein was prepared by replacing residues Asp321 and Asp323 of GnT-III by alanine residues (Ihara et al., 2002) (Fig. 1A). The spectrum of the glycans modified by the inactive chimeric protein indicates that the catalytic domain of GnT-III is mainly responsible for the oligosaccharide profile of the GnT-III^{ManII}-modified antibody. The reason for the minor increase in the fraction of complex non-fucosylated oligosaccharides needs further investigation (Table I). To ascertain if a different expression level of the enzymes may account for the differences in the oligosaccharide patterns, the expression of GnT-III was quantified by Western blot analysis via a C-terminal c-myc tag (Fig. 1B). Both GnT-III^{ManII} and GnT-III showed a slightly reduced expression compared to those of the other GnT-III chimeric proteins.

We also evaluated the hypothesis that the existence of relatively well organized functional glycosylation reaction subcompartments within the medial and trans Golgi cisternae may account for the glycosylation profiles derived from the chimeric GnT-III proteins. Given that pairs of charged amino acid residues in the stem regions of GnT-I and ManII have been postulated as critical for oligomer formation between enzymes (Nilsson et al., 1996), it was investigated if such a pairing could account for the GnT-III^{ManII}-derived antibody glycosylation profile. Therefore the amino acid mutations R73N, L79S, and E81S (GnT-III$^{mutManII(3aa)}$) or R60Q, R73N, L79S, and E81S (GnT-III$^{mutManII(4aa)}$) were introduced into the stem region of ManII (Fig. 1A). Both mutants were expressed in similar amounts (Fig. 1B) and yielded antibody glycovariants featuring substantially reduced proportions of bisected non-fucosylated oligosaccharides compared to the non-mutated GnT-III^{ManII} (Table I).

Either GnT-III^{ManII} or GnT-III was co-expressed with ManII to shift the biosynthetic pathway from hybrid to complex bisected oligosaccharides (Fig. 2B). The expression of both enzyme combinations lead to the generation of antibodies characterized by high proportions of complex type glycans lacking core-fucosylation, with the majority being bisected (Table I). In vitro galactosylation analysis confirmed that peaks at m/z 1,339 and 1,502, which were not digested by EndoH, could be assigned to non-fucosylated complex glycan structures, with only minor contribution of bisected non-fucosylated hybrid structures (Fig. 2, Table I). GnT-III^{ManII} was also co-expressed with GnT-II, an enzyme that similarly to ManII directs the glycosylation pathway toward the formation of complex type glycans. This led to the accumulation of high mannose structures and a low proportion of bisected non-fucosylated oligosaccharides (data not shown), and this enzyme combination was not investigated further.

Two glyco-engineered antibodies, namely those produced either by transient co-expression with GnT-III^{ManII} (termed "Glyco-1," bearing mainly hybrid non-fucosylated bisected glycans) or with GnT-III^{ManII} and ManII ("Glyco-2," bearing mainly complex non-fucosylated bisected glycans) were examined for their affinity for FcγRIIIa and compared either to the unmodified antibody or to an antibody glycovariant produced under the same conditions by transient co-expression of GnT-III ("Glyco-0") (Fig. 3).

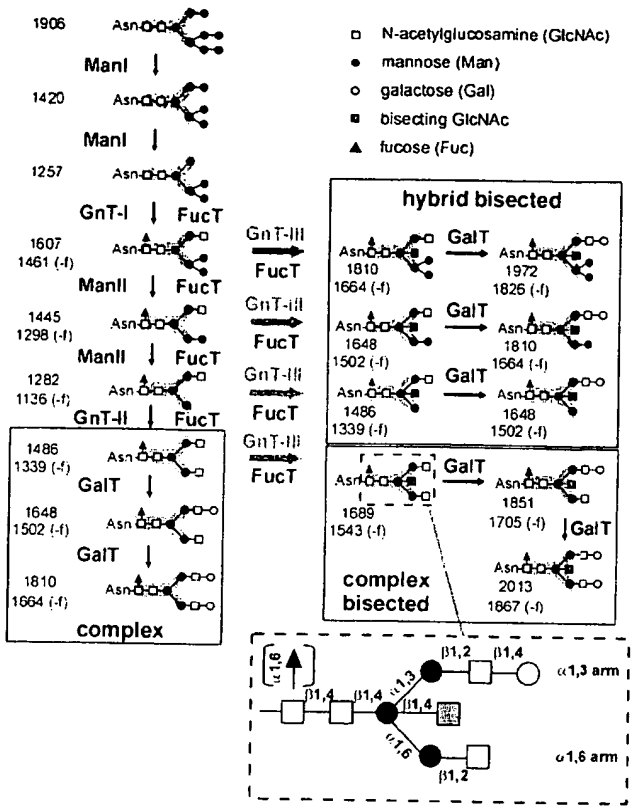

Figure 3. *N*-linked oligosaccharide biosynthetic pathway leading to complex or hybrid structures bearing a bisecting GlcNAc. The mass to charge (m/z) value of the sodium-associated oligosaccharide ion obtained by MALDI/TOF-MS analysis is indicated next to the corresponding oligosaccharide structure. The shaded monosaccharides belong to the core of the oligosaccharide, shared by all naturally occurring *N*-linked glycoforms, the presence of the other sugars is variable. A bisecting *N*-acetylglucosamine (GlcNAc; gray) can be β1,4 linked to the core mannose by the enzyme GnT-III, as long as the oligosaccharide has been previously modified by GnT-I. α1,6-fucosyltransferase (α1,6-FucT) catalyses the addition of a fucose α1,6-linked to the GlcNAc residue attached to Asn, to any oligosaccharides that have not been modified by GnT-III or GalT. Complex and hybrid glycans structures are defined by the structure of the α1,6 arm. ManI, mannosidase I; GnT-I, β1,2-*N*-acetylglucosaminyltransferase I; ManII, Golgi α-mannosidase II; GnT-II, β1,2-*N*-acetylglucosaminyltransferase II; GalT, β1,4-galactosyltransferase (GalT); GnT-III, β1,4-*N*-acetylglucosaminyltransferase III; α1,6-FucT, core α1,6-fucosyltransferase.

FcγRIIIa Binding of Glyco-0, Glyco-1, and Glyco-2 Anti-CD20 Monoclonal Antibodies

Binding of glyco-engineered antibodies to FcγRIIIa was evaluated on peripheral human natural killer (NK) cells, which are known to be important mediators of ADCC, and to constitutively express FcγRIIIa. Binding to NK cells was performed by incubating the antibody glycovariants with freshly isolated NK cells from a donor who was genotyped as heterozygous for FcγRIIIA-Val/Phe158 (Koene et al., 1997).

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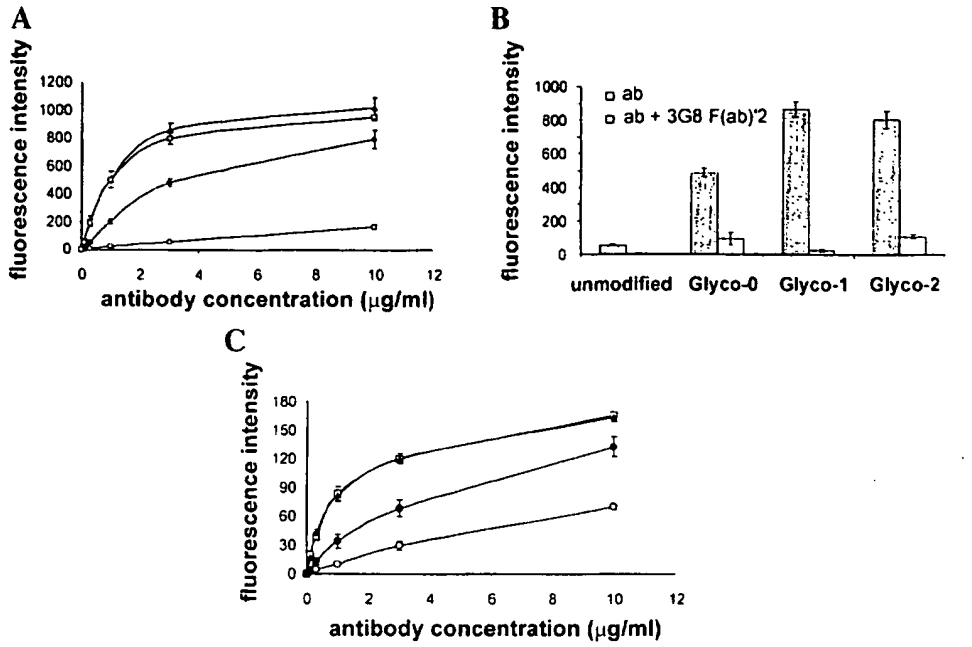

Figure 4. FcγRIIIa binding of monomeric anti-CD20 glycovariant antibodies. Binding to purified NK cells from a donor heterozygous for FcγRIIIA-Val/Phe 158 and negative for FcγRIIC was evaluated. **A**: Binding of antibodies at concentrations ranging from 0.1 to 10 µg/mL. **B**: Antibody binding (3 µg/mL) to FcγRIIIA was competed by addition of blocking anti-FcγRIIIa 3G8 F(ab')2 fragments (10 µg/mL). **C**: Binding to a CHO cell line stably expressing human FcγRIIIa-Val 158 α-chain and γ-chain was evaluated to confirm the dependence of antibody binding to FcγRIIIa. All assays were performed in quadruplicate. ○, unmodified; ●, Glyco-0; ▲, Glyco-1; and □, Glyco-2 antibodies.

Both Glyco-1 and Glyco-2 bind with a considerably higher affinity to NK cells than unmodified antibody (Fig. 4A). Under the transient gene expression levels of this study, GnT-III co-expression leads to an antibody (Glyco-0) with a lower level of bisected non-fucosylated oligosaccharides with intermediate FcγRIIIa binding affinity. Antibody binding to NK cells occurred exclusively via FcγRIIIa as it could be outcompeted by the addition of blocking anti-FcγRIIIa $F(ab')_2$ fragments (Fig. 4B). Similar results were obtained using a recombinant CHO cell line stably expressing the FcγRIIIa-Val158 α-chain receptor (Fig. 4C).

Biological Activity of Glyco-1 and Glyco-2 Anti-CD20 Monoclonal Antibodies

In a next step we investigated whether increased FcγRIIIa binding correlates with an improvement in the biological activity of the glyco-engineered antibodies, which are characterized by bisected non-fucosylated oligosaccharides. Both glycovariants mediate an enhanced ADCC against CD20-positive Raji cells, independently of the Fc-linked glycans being of complex or hybrid type (Fig. 5A).

Glyco-1 and Glyco-2 were also evaluated for complement-dependent cytotoxicity (CDC) against CD20-positive tumor cells SKW6.4 in the presence of human serum (Fig. 5B). Glyco-2, bearing complex oligosaccharides, performs similarly to the unmodified antibody in CDC. For Glyco-1, on the contrary, a reduction in CDC is observed, correlating with high proportion of hybrid oligosaccharides that are characteristic for this glycovariant.

To asses whether both ADCC and CDC can contribute to the elimination of target cells, we evaluated these glyco-engineered antibodies for B cell depletion in a whole blood assay (Fig. 5C). Glyco-1 and Glyco-2 anti-CD20 variants were over 100-fold more potent than unmodified anti-CD20 in depleting B cells, while no significant difference could be observed between the two glycovariants, indicating a minor contribution of CDC.

DISCUSSION

Chimeric GnT-III Proteins

GnT-III is an ideal enzyme to manipulate the *N*-glycosylation of expressed proteins (glyco-engineering), as it exerts a large degree of control over the glycosylation process by blocking the action of α1,6-FucT, ManII, and GnT-II (Schachter, 1986; Umaña et al., 1999). While natural human IgG in serum contains low percentages of bisected oligosaccharides in the Fc region (Wormald et al., 1997), up to 40% bisected glycans were observed for antibodies produced in YB2/0 rat myeloma cell line (Lifely et al., 1995). In the present study the impact of the localization domain of GnT-III on the antibody glycosylation profile was evaluated. We obtained antibodies with engineered carbohydrate moieties by co-expression of chimeric GnT-III proteins, composed of the catalytic domain of GnT-III fused to the localization domain of GnT-I, α1,6-FucT, GnT-II, or ManII (Fig. 1A). As for the GnT-III-derived antibody, the resulting glycovariants feature a bisecting *N*-acetylglucosamine (GlcNAc) residue on

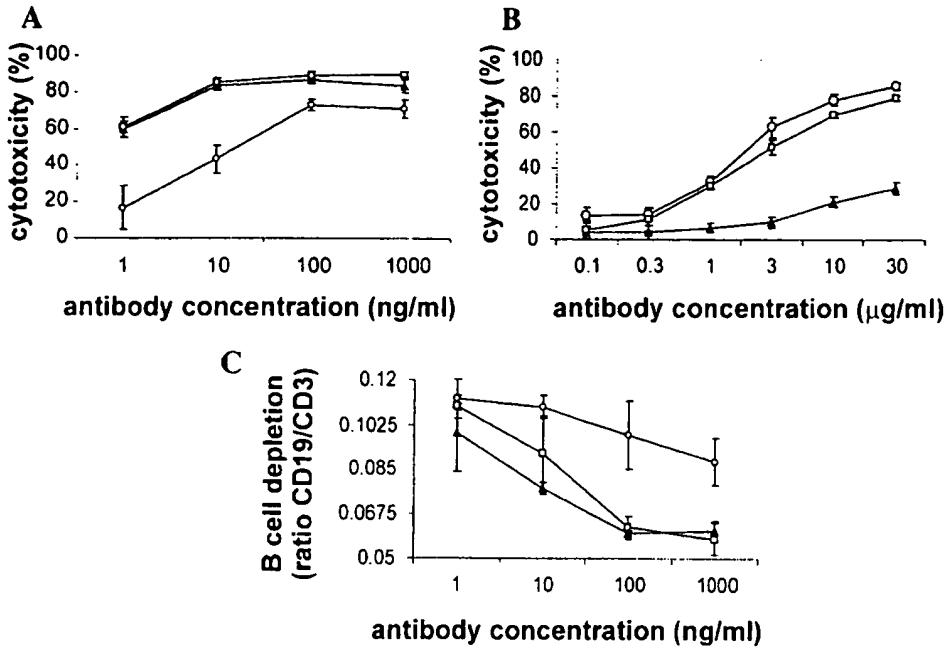

Figure 5. Biological activity assays of anti-CD20 antibody glycovariants. A: ADCC using PBMCs (FcγRIIIA-Val/Phe158, FcγRIIC negative donor) as effectors and human lymphoma Raji cells as targets. B: CDC against B lymphoblastoid SKW6.4 cells in the presence of human serum as a source of complement. C: B-cell depletion in whole blood (FcγRIIIA-Val/Phe158, FcγRIIC negative donor), which was calculated from the ratio of CD19-positive B cells to CD3-positive T cells as measured by FACS analysis. ○, unmodified; ▲, Glyco-1; and □, Glyco-2 antibodies.

almost all oligosaccharide structures, indicating that all chimeric GnT-III proteins were active (Table I, Fig. 2A). Relative to GnT-III-modified antibody, use of chimeric GnT-III led to an increase in the fractions of bisected non-fucosylated, and of bisected hybrid oligosaccharides, indicating a more efficient competition of the chimeric GnT-III against α1,6-FucT and ManII (Fig. 2A).

Among the chimeric GnT-III proteins, GnT-III^{ManII} is the most efficient in the competition against the above-mentioned enzymes, leading to the antibody glycovariant with the highest proportion of bisected non-fucosylated hybrid oligosaccharides. Since the expression level of GnT-III^{ManII} was not higher than for the other chimeric proteins or GnT-III, we propose that the higher efficiency of GnT-III^{ManII} results either from a different distribution in Golgi compartments or from a different functional organization of enzymes within a compartment. This allows GnT-III to act immediately after GnT-I in the biosynthetic process, leading to higher levels of bisected, non-fucosylated hybrid oligosaccharides (Table I) relative to the other chimeric GnT-III proteins.

Influence of the ManII Stem Region Residues on the Glycosylation Pattern

The higher efficiency of the chimeric protein GnT-III^{GnT-I}, compared to the unmodified GnT-III, for the synthesis of bisected hybrid and bisected non-fucosylated oligosaccharides can be explained by an earlier Golgi distribution, in the *cis*-to-*trans* direction of glycoprotein substrate transport, of GnT-I relative to GnT-III. The fine Golgi distributions of GnT-I and ManII have been determined previously by quantitative immunoelectron microscopy (Rabouille et al., 1995). Both enzymes co-distribute along the Golgi, being localized mainly in the medial and *trans* cisternae (Dunphy et al., 1985; Rabouille et al., 1995; Velasco et al., 1993). The spatial distributions of α1,6-FucT and GnT-II have not yet been determined quantitatively, but rat GnT-III has been found predominantly in the *trans* Golgi cisternae (Umaña, 1998). This, however, does not explain why the chimeric GnT-III^{ManII} is significantly more efficient than GnT-III^{GnT-I} at synthesizing bisected, hybrid, and bisected, non-fucosylated oligosaccharides, since both GnT-I and ManII have identical spatial distributions along the Golgi subcompartments. Additionally, the slightly lower expression level of GnT-III^{ManII} compared to those of the other chimeric GnT-III proteins, indicates that the antibody glycoprofile is not a result of an increased enzyme expression level (Fig. 1B), but a consequence of a more efficient processing of GnT-I-modified oligosaccharides, denoted here as "functional pairing" of GnT-III^{ManII} with GnT-I.

To assess if this functional pairing relies on a physical interaction between the two enzymes, we evaluated GnT-III^{ManII} mutants with amino acid substitutions in the localization domain of ManII which were reported to be critical determinants for the formation of hetero-oligomers of GnT-I and ManII (Nilsson et al., 1996). Although it has been suggested that these residues are not essential for incorporation into high molecular weight complexes of Golgi enzymes or even for Golgi localization (Opat et al., 2000), it is possible that they are involved in a finer pairing of the catalytic

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domains during oligosaccharide biosynthesis. We observed a significant reduction in bisected non-fucosylated hybrid oligosaccharides with these mutants (Table I). Both GnT-III^{mutManII} mutants were expressed at comparable levels as GnT-III^{ManII}, excluding the expression level of the latter as being responsible for higher proportions of bisected non-fucosylated glycans (Fig. 1B). However, the exchanged residues do not seem to be the sole determinant of the resulting oligosaccharide product distribution, suggesting either additional contributions of the rest of the stem or catalytic regions to functional enzyme pairing, or an enrichment of these enzymes in subcompartments caused by different mechanisms. Evidence for pairing may possibly be obtained via co-immunoprecipitation and electron microscopy experiments. Another possible explanation may be a conformational change caused by the modified stem region, which may lead to a catalytic domain with increased activity. Our data suggest that by virtue of the ManII localization domain, a physical and/or a functional pairing takes place between the catalytic domains of the endogenous GnT-I and the recombinant GnT-III^{ManII} chimeric protein.

Co-Expression of GnT-III^{ManII} and ManII

With the described approach, we are able to modulate the glycosylation pattern of antibodies from fucosylated complex glycans to bisected non-fucosylated hybrid oligosaccharides by overexpressing GnT-III^{ManII}. The co-expression of GnT-III^{ManII} and ManII, or of GnT-III and ManII led to the formation of bisected non-fucosylated glycans of the complex type. ManII overexpression redirects the biosynthetic pathway causing the product shift from hybrid to complex carbohydrates (Fig. 2B, Table I). Although equally high levels of bisected non-fucosylated complex oligosaccharides can be synthesized by high level expression of GnT-III, the results presented here show that GnT-III^{ManII} is more efficient at adding a bisecting GlcNAc residue to the GnT-I-processed oligosaccharides prior to the reactions catalyzed by ManII, GnT-II, and α1,6-FucT.

Similarly to ManII, GnT-II was co-expressed with GnT-III^{ManII} with the intent of forming complex type glycans linked to the antibody. The resulting glycovariant had lower proportions of bisected non-fucosylated (37%) and complex (22%) glycans compared to the GnT-III^{ManII}/ManII-derived antibody (data not shown). Moreover, a significant fraction of high mannose oligosaccharides characterized this glycovariant, suggesting an influence of the overexpression of GnT-II on the maturation process of high mannose glycans. Under these conditions, the enzyme seems to inhibit the GnT-I-mediated reaction (Table I) by unknown mechanisms. A similar phenomenon was reported for overexpression of β1,4-GalT, which led to an enrichment of high mannose oligosaccharides on recombinantly co-expressed IFN-γ (Fukuta et al., 2001). Although the cellular localization of GnT-II is yet to be discovered, high molecular weight complexes between GnT-I and GnT-II have been found in Golgi extracts of mammalian cells (Opat et al., 2000). The formation of such complexes might be disturbed by GnT-II overexpression.

We could show that the formation of a desired carbohydrate profile can be achieved by the combination and overexpression of enzymes, although not all the potential enzymes are suitable for this purpose, indicating that the glycosylation process is governed by a well-balanced system of enzymes that needs further elucidation.

Biological Activity of Glyco-Engineered Antibodies

A high affinity to FcγRIIIa is important for ADCC, which is mediated by unconjugated therapeutic antibodies in humans. This was deduced from pioneering pharmacogenomic studies evaluating the impact of the FcγRIIIA polymorphisms on the activity of rituximab in lymphoma patients (Cartron et al., 2002). In that study, the objective response rates at 2/12 months were 100/90% for homozygous FcγRIIIA-Val158 and 67/51% for FcγRIIIA-Phe158 carriers, respectively. The superior response of the former seems to be the result of a significantly increased binding of the antibody to FcγRIIIa-Val158 compared to FcγRIIIa-Phe158 (Koene et al., 1997).

Glyco-1, featuring mainly bisected non-fucosylated hybrid glycans, and Glyco-2, bearing mainly bisected non-fucosylated complex carbohydrates, were examined for their binding to FcγRIIIa and their reactivity in cytotoxicity assays. Both Glyco-1 and Glyco-2 have an increased affinity for FcγRIIIa, which correlates with high proportions of bisected non-fucosylated oligosaccharides but seems independent of the glycans being of the hybrid or complex type. It has been reported that the absence of core fucose is responsible for an increased affinity to FcγRIIIa (Shields et al., 2002). Moreover, the absence of core fucose but not the presence of galactose or bisecting GlcNAc was reported to be responsible for increased ADCC under the tested conditions (Shinkawa et al., 2003).

Both Glyco-1 and Glyco-2 mediate an increased ADCC to a similar extent compared to the unmodified antibody, but feature a different reactivity in CDC assays. While Glyco-2 acts similarly to the unmodified antibody, Glyco-1 displays a reduced CDC, suggesting a significant influence of the glycan type (complex vs. hybrid). The main difference between Glyco-1 and Glyco-2 is the structure of their carbohydrate α1,6-arm. In contrast to Glyco-1, carrying hybrid glycans with mannose residues α1,3- and α1,6-linked to the α-6 arm, Glyco-2 and unmodified antibodies have mainly β1,2-linked GlcNAc residues at this position (complex glycans), which may be followed by galactose. This carbohydrate arm is in close contact with the IgG-Cγ2 domain polypeptide and may therefore influence domain conformations required for binding to C1q (Duncan and Winter, 1988; Huber et al., 1976; Idusogie et al., 2000). An even larger reduction in CDC was reported for antibodies featuring only high mannose oligosaccharides (Wright and Morrison, 1994).

The glyco-engineered antibodies also performed better than their unmodified counterparts in the depletion of B-cells in a whole blood assay, where both ADCC and CDC contribute to the elimination of target cells. The reduction in CDC activity, observed for Glyco-1, does not seem to affect our model of B-cell depletion in whole blood, suggesting that ADCC is the predominant mechanism in this assay. Moreover as first-dose-related side effects in vivo have been recently attributed to complement activation (van der Kolk et al., 2001; Winkler et al., 1999), Glyco-1 may provide a tool to prevent these problems.

We could therefore demonstrate that apart from modulating glycosyltransferase expression levels, engineering of Golgi localization domains can also be exploited for the production of tailored glyco-engineered therapeutic antibodies with unique combinations of biological activities.

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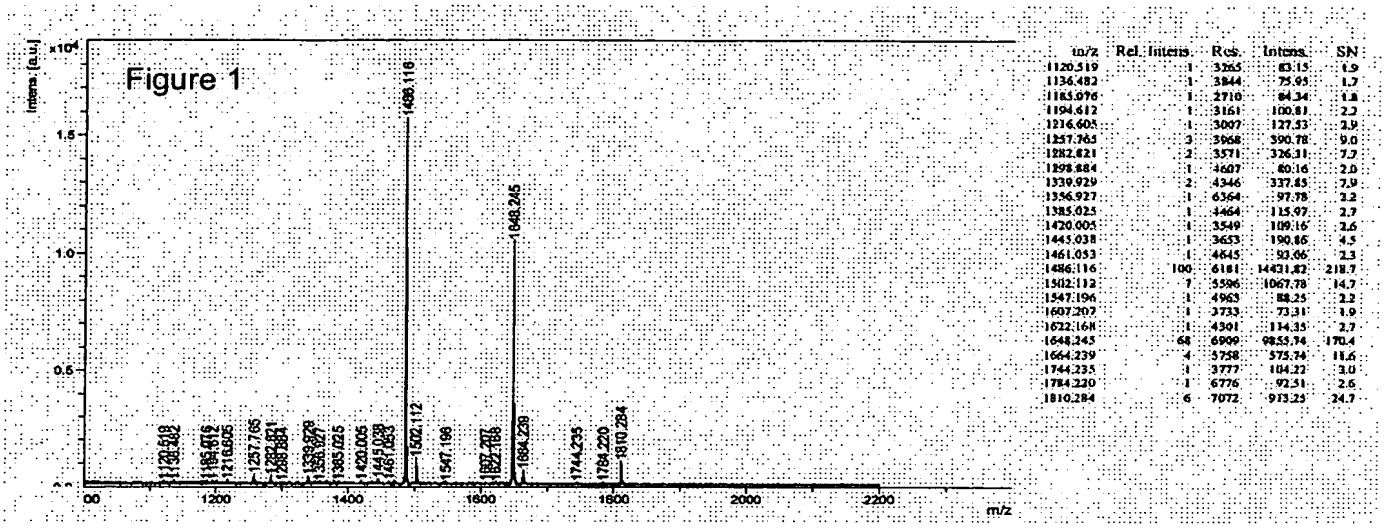
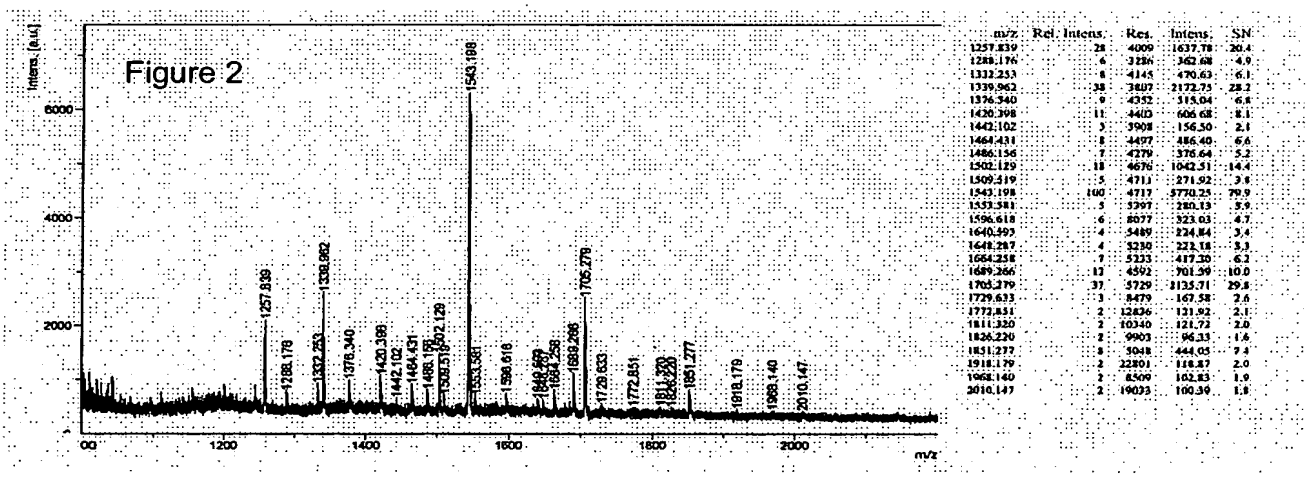
Antibody glycoengineering by constitutive co-expression of recombinant, wild-type β1,4-N-acetylglucosaminyltransferase III (GnT-III) and Golgi α-mannosidase II (ManII) in stable, industrial grade CHO cells producing a recombinant antibody



m/z	Rel. Intens.	Res.	Intens.	SN
1120.519	1	3265	83.15	1.9
1136.482	1	3844	75.95	1.7
1185.076	1	2710	84.34	1.8
1194.612	1	3161	100.81	2.2
1216.605	1	3007	127.53	2.9
1257.765	3	3968	390.78	9.0
1282.821	2	3571	326.31	7.7
1298.884	1	4607	80.16	2.0
1339.929	2	4346	337.85	7.9
1356.927	1	6364	97.78	2.2
1385.025	1	4464	115.97	2.7
1420.005	1	3549	109.16	2.6
1445.038	1	3653	190.86	4.5
1461.053	1	4845	93.06	2.3
1486.116	100	6181	14431.82	218.7
1502.112	7	5596	1067.78	14.7
1547.196	1	4963	88.25	2.2
1607.207	1	3733	73.31	1.9
1622.168	1	4301	134.35	2.7
1648.245	68	6909	9855.74	170.4
1664.239	4	5758	575.74	11.6
1744.235	1	3777	104.22	3.0
1784.220	1	6776	92.51	2.6
1810.284	6	7072	913.25	24.7

Figure 1 shows the glycosylation profile for the Fc-region oligosaccharides of a **non-glycoengineered recombinant antibody** produced in CHO cells. The three major peaks (m/z 1486, 1648 and 1810) correspond to fucosylated complex oligosaccharides, and level of non-fucosylated oligosaccharides is below 10%, which is typical for non-glycoengineered antibodies produced by CHO cells. The oligosaccharide profile was determined by MALDI/TOF-MS in positive ion mode for oligosaccharides enzymatically released from the antibody by PNGaseF treatment.



m/z	Rel. Intens.	Res.	Intens.	SN
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1332.253	8	4145	470.63	6.1
1339.962	38	3807	2172.75	28.2
1376.340	9	4352	515.04	6.8
1420.398	11	4403	606.68	8.1
1442.102	5	3908	156.50	2.1
1464.431	8	4497	486.40	6.6
1486.156	7	4279	376.64	5.2
1502.129	18	4676	1042.51	14.4
1509.519	5	4711	271.92	3.8
1543.198	100	4717	5770.25	79.9
1553.581	5	5397	280.13	3.9
1596.618	6	8077	323.03	4.7
1640.593	4	5489	224.84	3.4
1648.287	4	5230	223.18	3.3
1664.258	7	5233	417.20	6.2
1689.266	17	4592	701.59	10.0
1705.279	37	5729	2135.71	29.8
1729.633	3	8479	167.58	2.6
1772.851	2	12836	121.92	2.1
1811.320	2	10340	121.72	2.0
1826.220	2	9903	96.23	1.6
1851.277	8	3048	444.05	7.8
1918.179	2	22801	118.87	2.0
1968.140	2	8509	162.83	1.9
2010.147	2	19023	100.39	1.8

Figure 2 shows the glycosylation profile for the Fc-region oligosaccharides of a **glycoengineered recombinant antibody** produced in CHO cells. Glycoengineering was achieved by constitutive co-expression of recombinant, wild-type β1,4-N-acetylglucosaminyltransferase III (GnT-III) and Golgi α-mannosidase II (ManII) in stable, industrial grade CHO cells producing a recombinant antibody. Industrial grade means that the cells grow (with a doubling time lower than 40 hours) in suspension in chemically-defined protein-free medium and produce over 0.5 g/l of antibody under batch process conditions. Glycoengineering leads to increased levels of non-fucosylated oligosaccharides relative to levels produced in non-glycoengineered CHO cells (peaks at m/z 1339, 1543 and 1705 are

non-fucosylated complex oligosaccharides and represent over 70% of the oligosaccharides). The oligosaccharide profile was determined by MALDI/TOF-MS in positive ion mode for oligosaccharides enzymatically released from the antibody by PNGaseF treatment.

Figure 3 **Fc-FcR binding of antibodies to CHO cells stably expressing recombinant human FcgRIII**

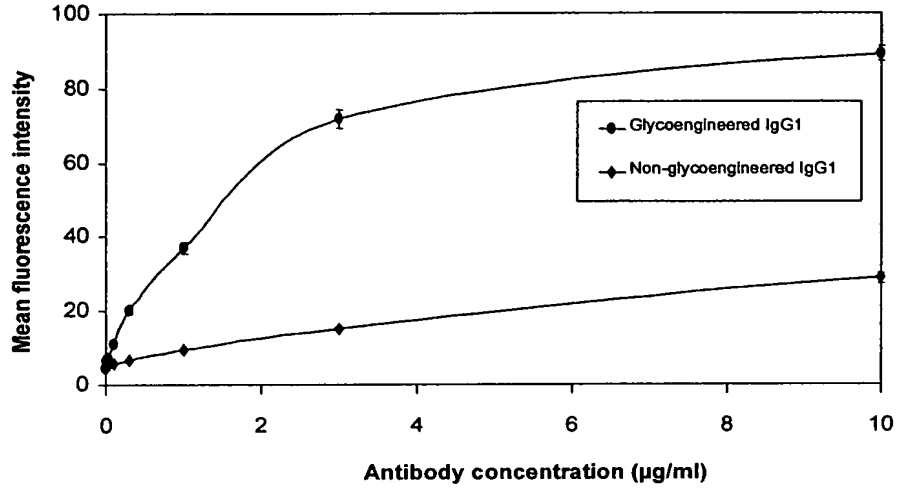


Figure 3 shows increased binding to FcgRIII for a glycoengineered antibody, having increased levels of non-fucosylated Fc-oligosaccharides (glycoengineering as described above for Figure 2), relative to a non-glycoengineered antibody with identical Fc polypeptide region. Binding assay was performed as described in .

Figure 4 **Increased biological activity (antibody dependent killing of B-cells in whole blood) by a glycoengineered antibody with increased levels of non-fucosylated oligosaccharides**

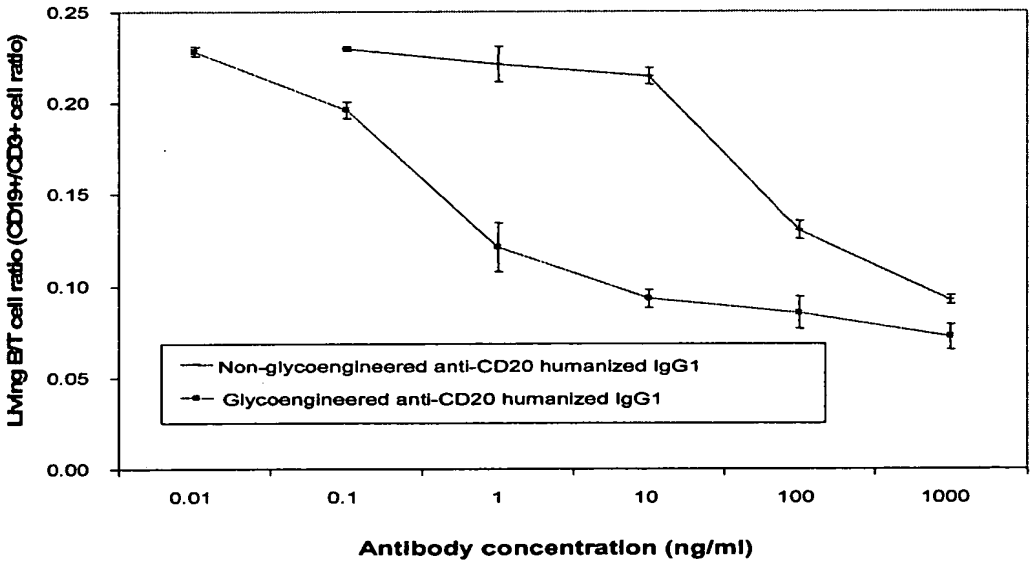


Figure 4 shows increased antibody-dependent killing of target cells for a glycoengineered antibody with increased levels of non-fucosylated Fc-oligosaccharides (glycoengineered as

described above for Figure 2) relative to a non-glycoengineered antibody with identical Fc polypeptide region. B-cell antibody-dependent-depletion assay performed by incubating antibodies with whole blood overnight and measuring the levels of remaining, living B-cells by flow cytometry (T-cells are used as an internal standard that is not depleted by the anti-CD20 antibodies). Assay details are as described in .

More specifically, the present invention is directed to a method for producing an altered glycoforms of proteins having improved therapeutic values, *e.g.*, an antibody which has an enhanced antibody dependent cellular cytotoxicity (ADCC), in a host cell. The invention provides host cells which harbor a nucleic acid encoding the protein of interest, *e.g.*, an antibody, and at least one nucleic acid encoding a glycosyl transferase. Further, the present invention provides methods and protocols of culturing such host cells under conditions which permit the expression of said protein of interest, *e.g.*, the antibody having enhanced antibody dependent cellular cytotoxicity. Further, methods for isolating the so generated protein having an altered glycosylation pattern, *e.g.*, the antibody with enhanced antibody dependent cellular cytotoxicity, are described.

Furthermore, the present invention provides alternative glycoforms of proteins having improved therapeutic properties. The proteins of the invention include antibodies with an enhanced antibody-dependent cellular cytotoxicity (ADCC), which have been generated using the disclosed methods and host cells.

IV. BRIEF DESCRIPTION OF THE DRAWINGS

FIGURE 1 depicts structures of common types of N-linked oligosaccharides and nomenclature. M stands for mannose; Gn, N-acetylglucosamine (GlcNAc); G, galactose; Gn^b, bisecting GlcNAc; R, Asn-Gn-β1,4-Gn or Asn-Gn-β1,4-(α1,6-fucose)-Gn. R-M is called the oligosaccharide "core". The square brackets indicated that at least one Gn is linked to a G. the oligosaccharide nomenclature used in this work consists of enumerating the M and Gn residues attached to the R group, indicating the presence of a bisecting GlcNAc by including a Gn^b, and indicating if the oligosaccharide is galactosylated by including a G. The two types of tri-antennary oligosaccharides are differentiated by addint an apostrophe to the Gn_3 term.

FIGURE 2 depicts central reaction network on the N-linked glycosylation pathway. This set of Golgi-localized reactions determines the major types of structures into which N-linked oligosaccharides are normally classified. The enzyme catalyzing each reaction is shown and all reactions have been numbered. The reaction numbers are used to denote the kinetic parameters associated with each reaction (*see*, TABLE IV for example).

B. A Mathematical Model Of N-Linked Glycoform Biosynthesis

1. Overview

Metabolic engineering of N-linked oligosaccharide biosynthesis to produce novel glycoforms or glycoform distributions of a recombinant glycoprotein can potentially lead to an improved therapeutic performance of the glycoprotein product. Effect engineering of this pathway to maximize the fractions of beneficial glycoforms within the glycoform population of a target glycoprotein can be aided by a mathematical model of the N-linked glycosylation process. A mathematical model is presented here, whose main function is to calculate the expected qualitative trends in the N-linked oligosaccharide distribution resulting from changes in the levels of one or more enzymes involved in the network of enzyme-catalyzed reactions which accomplish N-linked oligosaccharide biosynthesis. It consists of mass balances for 33 different oligosaccharide species N-linked to a specific protein that is being transported through the different compartments of the Golgi complex. Values of the model parameters describing Chinese hamster ovary (CHO) cells were estimated from literature information. A basal set of kinetic parameters for the enzyme-catalyzed reactions acting on free oligosaccharide substrates was also obtained from the literature. The solution of the system for this basal set of parameters gave a glycoform distribution consisting mainly of complex-galactosylated oligosaccharides, distributed in structures with different numbers of antennae in a fashion similar to that observed for various recombinant proteins produced in CHO cells. Other simulations indicate that changes in the oligosaccharide distribution could easily result from alteration in glycoprotein productivity within the range currently attainable in industry. The overexpress of N-acetylglucosaminyltransferase III (GnT III) in CHO cells was simulated under different conditions to test the main function of the model. These simulations allow a comparison of different strategies, such as simultaneous overexpression of several enzymes or spatial relocation of enzymes, when trying to optimize a particular glycoform distribution.

The mathematical model disclosed herein consists of mass balances for 33 different oligosaccharide species N-linked to a specified protein that is being transported through the different compartments of the Golgi complex. These equations relate the oligosaccharide mole fractions to the amounts of the different enzymes, the kinetic

constants of the reactions, the distribution of enzymes in the different compartments, the half-life of the protein in the Golgi, the volume of the compartments, and the specific glycoprotein productivity. Values for the parameters in the model and their normal ranges can either be found in the literature or estimated from literature information. Some of the parameters are specific for each cell line. Those describing Chinese hamster ovary (CHO) cells were used here, since CHO cells are currently the most common host for the industrial production of therapeutic glycoproteins. Numerical simulations of the model with these values of the parameters gave glycoform distributions similar to those observed for some proteins produced in CHO cells.

One characteristic of the glycosylation pathway makes its modelling different from that of other biochemical pathways. Oligosaccharides have some degree of conformational flexibility and, through interactions with the polypeptide chain, certain conformations can be preferentially stabilized. Wyss and Gerhard, 1996, *Current Opinion Biotechnol.* 7:409-416. In addition, the polypeptide backbone around the glycosylation site may limit the access of the catalytic sites of the enzymes to the oligosaccharide. Shao and Wold, 1995, *Eur. J. Biochem.* 228:79-85. As a result, a particular glycosylation site can have its own set of values for the kinetic constants of the enzyme-catalyzed reactions. These values can be different from those of other glycosylation sites in the same or other proteins. The occurrence of this phenomenon can be inferred from the numerous examples where very different oligosaccharide distributions have been observed for different glycosylation sites of the same protein, even though all other system variables were identical for all sites during biosynthesis. Nevertheless, the range of values of the kinetic constants for oligosaccharides on some glycoproteins lies close to the corresponding range for soluble oligosaccharides (Do *et al.*, 1994, *J. Biol. Chem.* 269:23456-23464; Rao and Mendicino, 1978, *Biochemistry* 17:5635-5638; Gross *et al.*, 1990, *Anal. Biochem.* 186:127-134. Motivated by this observation, the constants for the latter were used as an initial approach to test the model and to study some aspects of its general behavior.

2. Physical Model

The N-linked glycosylation pathway consists of enzyme-catalyzed reactions which first attach a common oligosaccharide precursor to appropriate glycosylation sites in a polypeptide and then modify the linked oligosaccharides to produce a heterogenous set of glycoforms. Kornfeld and Kornfeld, 1985, *Annu. Rev. Biochem.* 54:631-664. Potential glycosylation sites are asparagine residues in the sequence Asn-X-Ser/Thr. The reactions take place in the endoplasmic reticulum (ER) and in the Golgi complex (Golgi) as proteins are transported through these cellular compartments *en route* to their final destinations. These destinations may be, for example, the ER or Golgi themselves, the plasma membrane, or the extracellular space.

The initial covalent attachment of the oligosaccharide precursor to the protein takes place during translocation of the latter into the lumen of the ER. Not all the translocated molecules acquire oligosaccharides in their potential glycosylation sites, and the fraction that does may vary between sites. The type of glycoform heterogeneity which thus results is called glycoform macro-heterogeneity. Shelikoff *et al.*, 1996, *Biotechnol. Bioeng.* 50:73-90. Once in the ER, the N-linked oligosaccharides are trimmed down by glycosidases which can sequentially remove three molecules of glucose and, sometimes, one of mannose. The glycoproteins are then transported to the Golgi where a different set of glycosidases and glycosyltransferases act on the N-linked oligosaccharides and lead to a diversity of structures. Such type of heterogeneity in the identity of the attached oligosaccharides is referred to as glycoform micro-heterogeneity.

A mathematical model of glycoform macro-heterogeneity has been published recently. Shelikoff *et al.*, 1996, *supra*. It incorporates different factors that determine the extent of the first transfer reaction of the pathway. In contrast, the model presented below is concerned with glycoform micro-heterogeneity. More specifically, it deals with a set of eight Golgi-localized enzymes which together determine the distribution of oligosaccharides into the following major structural classes: high mannose, hybrid, bi-, tri-, tri'- and tetra-antennary complex, bisected hybrid, and bisected bi-, tri-, tri'-, and tetra-antennary complex oligosaccharides (FIGURES 1 and 2). Thirty-three different oligosaccharide species are involved in 33 reactions catalyzed by these enzymes; including 5 high-mannose, 3 hybrid, 3 hybrid-galactosylated, 4 complex (bi-, tri-, tri',

and tetra-antennary complex), 4 complex-galactosylated, and the 14 bisected counterparts of the hybrid and complex oligosaccharides. The products of this set of reactions can be processed further in the Golgi through more transferase-catalyzed reactions that increase glycoform micro-heterogeneity.

The major elements of the physical model are: (a) the different Golgi compartments where the reactions take place and the transport of proteins between them, (b) the central network of enzyme-catalyzed reactions, and (c) the spatial distribution of these enzymes in the different Golgi compartments.

Golgi Compartments. The Golgi complex consists of a series of distinct, membrane-bounded compartments. Proteins destined to the extracellular space, plasma membrane, lysosomes, endosomes, or secretory storage fesicles are transported from the ER to the first Golgi compartment, the cis-Golgi network (CGN). From there, they travel in sequential order through the remaining compartments of the series; the cis-medial-, and trans-Golgi cisternae, which together comprise the Golgi stack; and then to the trans-Golgi network (TGN), the final sorting place. Rothman and Orci, 1992, *Nature* 355:409-415. There is some controversy about the number of cisternae in the Golgi stack, but in the present model only three are considered.

Proteins are transported between compartments by vesicles which bud off from the membrane of one compartment and fuse to the next in the series. Rothman and Wieland, 1996, *Science* 272:227-234. Secreted and plasma membrane proteins appear to go through the Golgi by a "bulk flow" mechanism. These proteins enter vesicles by default, *i.e.*, in the absence of specific transport or retention signals, and therefore at their bulk concentration in the donor compartment. Proteins which reside in the ER or Golgi require retention signals that allow them to be concentrated in the appropriate compartments. Such residency is not permanent and their relative concentration in a particular region is also aided by retrieval-vesicles that recognize transport signals in escaped proteins and return them to previous compartments.

For the physical model, four of the five Golgi compartments mentioned above are considered as a system of four reactors in series. The modelled compartments are the cis-, medial-, and trans-Golgi cisternae, and the TGN. This selection is based on immuno-electron microscopy studies that localize the enzymes included in the present

model to these compartments. Nilsson *et al.*, 1993, *J. Cell. Biol.* 120:5-13; Rabouille *et al.*, 1995, *J. Cell Science* 108:1617-1627. The chemical reactions catalyzed by these enzymes are described next.

Central Reaction-Network. The N-linked glycosylation pathway of mammalian cells has been deduced by a combination of *in vitro* and *in vivo* biosynthetic studies. Kornfeld and Kornfeld, 1985, *supra*; Schachter, 1986, *Biochem. Cell Biol.* 64:163-181. Although many enzymes participate in the pathway, a subset of them determines the distribution of oligosaccharides into 33 different species which together define the high mannose, hybrid, hybrid-bisected, complex and complex-bisected types. The network of reactions catalyzed by this subset is called the "central reaction network" (CRN). The CRN considered in the present model is depicted in FIGURE 2.

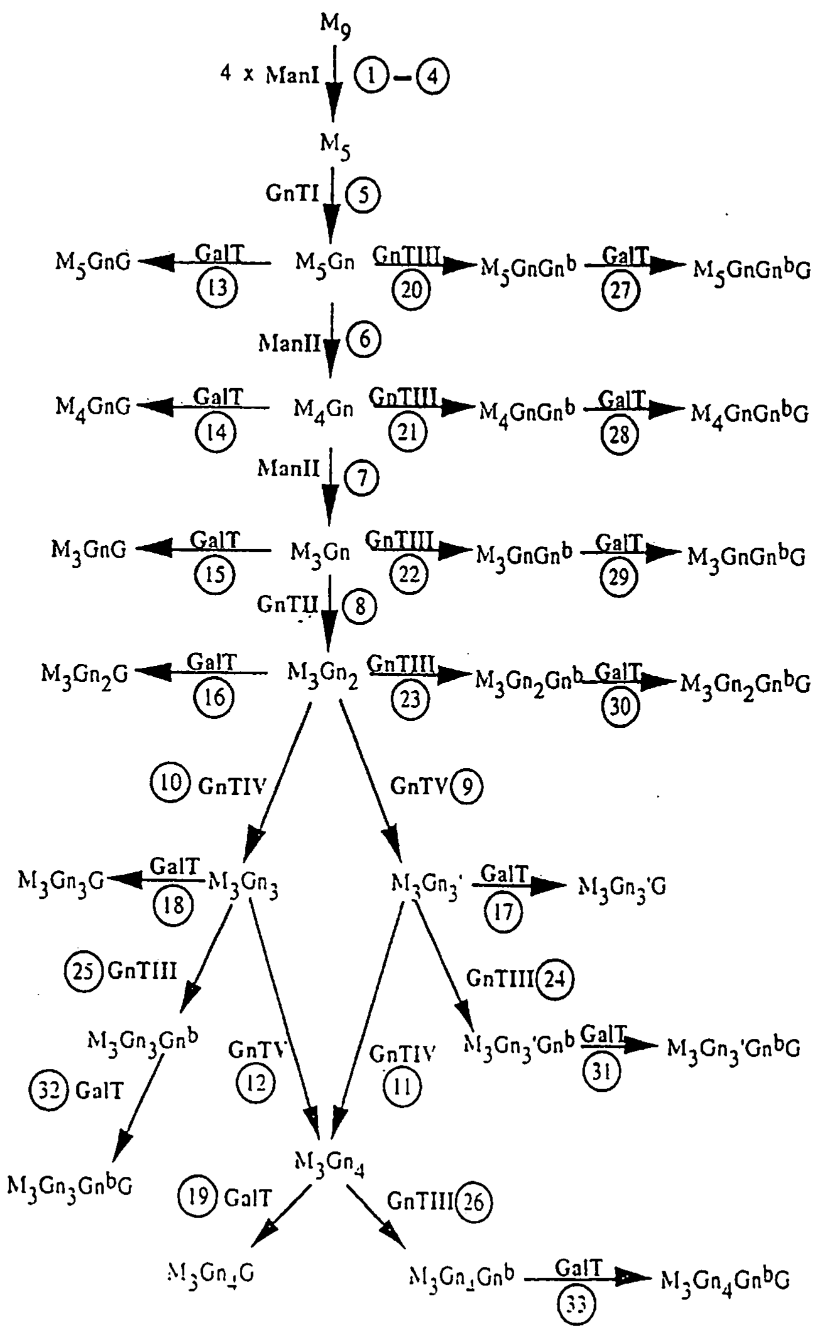
The first enzyme of the CRN is Golgi α1,2-mannosidase I (Man I), which can cleave α1,2-linked mannose residues from M_9-M_6 to finally produce M_5 (*see*, nomenclature in FIGURE 1), corresponding to reactions 1 to 4 in FIGURE 2. All eukaryotic cells have an α1,2-mannosidase in the ER that can also catalyze reaction 1. Therefore, the initial substrate for the Golgi CRN is a mixture of M_9 and M_8 oligosaccharides. Compounds M_9 to M_5 constitute the high-mannose class of N-linked oligosaccharides. The synthesis of hybrid and complex oligosaccharides then follows as described above.

An N-acetylglucosamine (glCNAc) can be transferred to the α1,3-mannose branch of M_5 by β1,2-N-acetylglucosaminyltransferase I (GnT I) to yield M_5Gn, the first hybrid oligosaccharide. M_5Gn is a substrate for α-mannosidase II (Man II), which catalyzes the removal of two mannose residues resulting in hybrids M_4Gn (Reaction 6) and M_3Gn (reaction 7). The free α1,6-mannose branch of M_3Gn is then available for extension by GnT II to produce M_3Gn_2, a complex bi-antennary oligosaccharide. M_3Gn_2 may be branched further by GnT IV or GnT V. GnT IV adds a GlcNAc in a β1,4-linkage to the α1,6-mannose branch, leading to the tri-antennary complex oligosaccharide M_3Gn_3. GnT V catalyzes a GlcNAc transfer in aβ1,6-linkage to the α1,3-mannose branch and produces the tri'=antennary complex oligosaccharide M_3Gn_3'. The tetra-antennary complex compound M_3Gn_4 can be synthesized both by GnT IV from M_3Gn_3' (reaction 11) and by GnT V from M_3Gn_3 (reaction 12).

All hybrid and complex oligosaccharides contain non-reducing-end GlcNAcs which may be extended by β1,4-galactosyltransferase (GalT, reaction 13 to 19). Once a galactose residue is transferred, the modified oligosaccharide is no longer a biosynthetic substrate for any of the remaining GnTs or for Man II. Schachter, 1986, *supra*. All of the branches in any complex oligosaccharide serve as substrates for GalT, but do so with different affinities. Pacquet *et al.*, 1984, *J. Biol. Chem.* 259:4716-4721. In the present model, these reactions are lumped together in single steps which remove the compound from the flux through reactions 1 to 12.

The reactions mentioned to this point take place in common industrial cell lines, such as CHO cells and baby hamster kidney (BHK) cells, used for the production of recombinant glycoproteins. Jenkins *et al.*, 1996, *Nature Bioltechnol.* 14:975-981. An additional set of reactions (20 to 33) is also important for determining the major classes of N-linked oligosaccharides in cell lines expressing GnT III. Examples of these cell lines are a glycosylation mutant of CHO cells named Lec 10 (Stanley and Campbell, 1984, *J. Biol. Chem.* 261:13370-13378) and rate myeloma (Y0) cells (Lifely *et al.*, 1995, *Glycobiology* 318:813-822. As indicated in reactions 20 to 26, GnT III can modify any non-galactosylated hybrid or complex oligosaccharide by transferring a GlcNAc residue in a β-1,4-linkage to the core mannose. The transferred residue is called a bisecting GlcNAc (Gn^b), and the products of these reactions are referred to as bisected oligosaccharides. GalT cannot extend the Gn^b residue, but it may modify all the other non-reducing-end GlcNAcs of any bisected oligosaccharide (reactions 27 to 33).

The final products of the CRN are usually modified further in the Golgi by the addition of sialic acids, poly-N-acetyllactosamine, fucose, N-acetylgalactosamine, sulphate, and α1,3-linked galactose. Wild type CHO cells only add sialic acids (in α2,3-linkages to galactose), fucose (α1,6-linked to the oligosaccharide core, *see*, R in FIGURE 1), and poly-N-acetyllactosamine (to various antennae but preferentially to that synthesized by GnT V). The addition of fucose to the core of oligosaccharides can take place at any point after reaction 5 of the CRN,b ut it is also blocked by the modifications that GalT or GnT III introduce. Core-fucosylated oligosaccharides can go through the rest of the CRN in the same way as their non-fucosylated counterparts,


M9
4 x ManI
1 – 4
M5
GnTI
5
M5GnG
GalT
13
M5Gn
GnTIII
20
M5GnGnb
GalT
27
M5GnGnbG
ManII
6
M4GnG
GalT
14
M4Gn
GnTIII
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10 GnTIV
GnTV 9
M3Gn3G
GalT
18
M3Gn3
M3Gn3'
GalT
17
M3Gn3'G
25 GnTIII
GnTIII 24
M3Gn3Gnb
GnTV
12
GnTIV
11
M3Gn3'Gnb
GalT
31
M3Gn3'GnbG
32 GalT
M3Gn3GnbG
M3Gn4
19 GalT
GnTIII 26
M3Gn4G
M3Gn4Gnb
GalT
33
M3Gn4GnbG

FIGURE 2

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Alteration of Oligosaccharide Biosynthesis by Genetic Manipulation of Glycosyltransferases[a]

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INTRODUCTION

Carbohydrate chains can directly mediate or modulate the function of glycoproteins in diverse biological processes.[1] The ability to manipulate the oligosaccharide structures of glycoproteins in order to alter their biological properties would be of obvious value. This is particularly relevant with regard to biologically important molecules such as growth factors, hormones, and other therapeutic agents that are being produced in cultured cells. Altering the sugar chains of these glycoproteins may improve their therapeutic value by increasing their efficacy, altering their circulatory half-lives, and/or increasing their target specificity. In addition, altered glycosylation of cell-surface components may provide insight to the precise roles that cell-surface glycoconjugates play in processes such as migration, adhesion, development, and malignancy.

Several methods have evolved to alter glycosylation in cells. These have included the use of reagents that inhibit glycosylation as well as inhibitors of glycosylation processing.[2] These inhibitors have been widely used to study the sugar chains of glycoconjugates, but many of these reagents are toxic to cells, and in some instances, their effects are only partial. Another approach has been to use mutagenized cells that are resistant to the toxic effects of specific lectins due to deficiencies in corresponding glycosylation reactions.[3] One limitation of this approach is that mutants are not generated at each step of the biosynthetic pathway. In addition, both of these approaches are of limited use for obtaining large, complex oligosaccharides, inasmuch as they result in the formation of incomplete or truncated carbohydrate structures.

One method that overcomes these limitations and that allows one to selectively manipulate oligosaccharide structure is to express cloned genes for glycosyltransferases into mammalian cells. As more genes for these enzymes are cloned, the possibilities for altering the biosynthetic pathways of oligosaccharide in cells could be substantial. It is this new approach that will be the focus of this review.

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GLYCOSYLTRANSFERASES

Glycosyltransferases are enzymes localized to the endoplasmic reticulum and Golgi complex of all mammalian cells. These enzymes catalyze the transfer of sugars donated from either lipid or nucleotide intermediates to mono- and oligosaccharide acceptors. To date, more than a dozen different glycosyltransferases have been cloned and characterized.[4] Although some of these enzymes share common substrates, they show very little sequence homology. The topology and domain structures of all the cloned glycosyltransferases, however, are remarkably similar in that all are type II membrane glycoproteins containing short cytoplasmic domains and large lumenal catalytic domains. The availability of cDNAs for many of these enzymes makes it possible to reintroduce them into host cells and to subsequently affect glycosylation.

Alteration of glycosylation through genetic manipulation of glycosyltransferases can be classified into two categories: the expression of glycosyltransferases that are not normally active in the host cells (exogenous glycosyltransferases), and overexpression of glycosyltransferases in host cells containing endogenous activities of these enzymes (endogenous glycosyltransferases). The first approach has been the most extensively studied because screening for alterations in glycosylation is relatively simple, requiring the detection of novel oligosaccharides on the cell surface using antibody and/or lectin probes. The second approach, on the other hand, requires more rigorous quantitative analysis because increasing the levels of glycosyltransferases would most likely not generate different types, but rather, different amounts of oligosaccharide structures.

GENETIC MANIPULATION OF GLYCOSYLTRANSFERASE EXPRESSION

Exogenous Glycosyltransferases

With few exceptions, the introduction of exogenous glycosyltransferases has used enzymes that act on terminal glycosylation, such as fucosylation and sialylation. The following section describes some studies that have used this approach to modify glycosylation.

A. α1,3 Fucosyltransferase (α1,3 FT) catalyzes the transfer of fucose (Fuc) from GDP-fucose to *N*-acetylglucosamine (GlcNAc) of an *N*-acetyllactosamine unit. Several reports have demonstrated that introducing the cDNA for this glycosyltransferase into Chinese hamster ovary (CHO) cells lacking this activity results in the biosynthesis of fucosylated *N*-acetyllactosamine, also known as the Lewis x (Le^x) antigen.[5–7] This oligosaccharide is of biological interest because it is the ligand for the ELAM receptor on endothelial cells and is required for lymphocyte homing. The ability to synthesize large quantities of oligosaccharides and glycoproteins containing Le^x is of considerable interest, because these oligosaccharides may have potential therapeutic applications in the treatment of inflammation[8] and, perhaps, malignant invasion.[9]

B. α1,2 Fucosyltransferase (α1,2 FT) catalyzes the transfer of Fuc from GDP-

fucose to galactose (Gal) residues, resulting in the formation of the H blood group antigen. Similar to the studies with α1,3 FT, the introduction of the cloned gene for α1,2 FT into COS-1 cells, which lack this enzymic activity, results in the formation of H blood group antigen.[10]

C. α1,3 Galactosyltransferase (α1,3 GT) catalyzes the transfer of Gal from UDP-Gal to terminal Gal residues of oligosaccharide chains. Transfection of α1,3 GT into CHO cells results in the expression of α1,3 Gal-containing oligosaccharides.[11] Furthermore, the expression of this enzyme results in a concomitant decrease in terminal sialylation of sugar chains in these cells. Thus, this study also shows that competition between different glycosyltransferases can affect glycosylation *in vivo*.

D. α2,6 Sialyltransferase (α2,6 ST) catalyzes the transfer of sialic acid (SA) from CMP-SA to terminal Gal residues. Transfection of the cDNA encoding this protein into CHO cells results in the expression of appropriately sialylated sugar chains.[12] As was the case for example C above, there is competition for substrates between the transfected sialyltransferase and a similar endogenous enzyme that adds SA in an α2,3 linkage to Gal.

E. β1,3 Galactosyltransferase (β1,3 GT) catalyzes the transfer of Gal to terminal GlcNAc residues in the biosynthesis of type 1 (Gal β1,3 GlcNAc) *N*-acetyllactosamine chains. This class of oligosaccharides is of particular biological and structural importance because it serves as the core structure for various blood group activities. Transfection of the cDNA encoding this enzyme into human colonic cells results in the biosynthesis of type 1 *N*-acetyllactosamine-containing oligosaccharides.[13] In addition, many of these chains were further modified with SA and Fuc residues. In contrast to the studies described above, this study is unique in showing that the expression of glycosyltransferase acting early in oligosaccharide biosynthesis (*i.e.*, during elongation) can also alter glycosylation.

Endogenous Glycosyltransferase

To date, the only example of over-expression of an endogenously expressed glycosyltransferase is that of β1,4 galactosyltransferase (β1,4 GT). β1,4 GT catalyzes the transfer of Gal from UDP-Gal to terminal GlcNAc residues in the biosynthesis of type 2 (Gal β1,4 GlcNAc) *N*-acetyllactosamine cores of all *N*-linked complex oligosaccharides. β1,4 GT is unique among the cloned glycosyltransferases in that one gene encodes two similar forms of the enzyme that differ by an additional 13 amino acids at the cytoplasmic N-terminus of the long form that is not present in the short form of the enzyme.[14,15] Both the long and the short form are localized primarily in the Golgi complex. However, the long form of GT is also targeted to the plasma membrane,[15] where it associates with the cytoskeleton and functions as a cell-adhesion molecule.[16]

Because of the central role this enzyme plays in the biosynthesis of *N*-acetyllactosamine–containing oligosaccharides, the effects of overexpressing this enzyme were explored in detail. Transfection of the cDNAs encoding the two forms of β1,4 GT into F9 embryonal carcinoma cells expressing endogenous enzyme results in a threefold increase in total β1,4 GT activity compared to control cells.[17] Analysis of [^{3}H]Gal-labeled glycoproteins and glycopeptides by a variety of methods revealed

no significant quantitative or qualitative differences in glycosylation. Similar analysis of a specific glycoprotein, lysosomal-associated membrane glycoprotein (LAMP)-1 indicated no differences in glycosylation between the transfected and control cell lines. The inability of transfected β1,4 GT to affect glycosylation was not due to an absence of GT substrates, inasmuch as an excess of substrate was detected in lysed cells using endogenous and exogenous GT. The transfected GT were properly targeted to the Golgi complex, and, more importantly, were elevated in this compartment in the transfected cells. Thus, in these cells, β1,4 GT is not rate limiting for oligosaccharide biosynthesis.

SUMMARY

The alteration of oligosaccharide structures through genetic manipulation of glycosyltransferase activities is now a reality. It is apparent that this technique has greater consequences on oligosaccharide structure when an exogenous enzyme is introduced into cells, and in particular when this enzyme is responsible for a terminal glycosylation step. By contrast, only one study has examined the effects of overexpressing an endogenous glycosyltransferase, in which there was no detectable effect on glycosylation. However, there are still other key regulatory biosynthetic enzymes, such as GlcNAc transferase V and β1,3 GlcNAc transferase, whose overexpression may alter glycosylation. Both of these enzymes are required for the biosynthesis of polylactosaminoglycans (polymers of *N*-acetyllactosamine disaccharides), and their elevation in tumor cells correlates with increased expression of polylactosaminoglycans.[18,19] Recently, the gene encoding GlcNAc transferase V has been isolated,[20] but its transfection into cells and characterization of the resulting oligosaccharides awaits further study.

Alternate strategies for modifying oligosaccharide structures could involve the introduction of more than one glycosyltransferase into cells to ensure the availability of biosynthetic intermediates. Alternatively, the disruption of specific glycosyltransferase genes by homologous recombination could be used to eliminate competing glycosyltransferases that act on a common substrate.

Although oligosaccharide biosynthesis is directly dependent upon the presence or absence of specific glycosyltransferases, other factors also contribute to glycosylation. For example, the transport rate of a glycoprotein through the endoplasmic reticulum and Golgi complex, the levels of processing glycosidases, the availability of substrates, the host cell, and ultimately, the peptide backbone of the particular glycoprotein of interest are important contributors to the final outcome of oligosaccharide structure. Despite these complications, further study into the manipulation of glycosyltransferase genes may ultimately allow the controlled and predictable biosynthesis of glycoprotein sugar chains.

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Genetic engineering of recombinant glycoproteins and the glycosylation pathway in mammalian host cells

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The analysis of many natural glycoproteins and their recombinant counterparts from mammalian hosts has revealed that the basic oligosaccharide structures and the site occupancy of glycosylated polypeptides are primarily dictated by the protein conformation.

The equipment of many frequently used host cells (e.g. BHK-21 and CHO-cells) with glycosyltransferases, nucleotide-sugar synthases and transporters appears to be sufficient to guarantee complex-type glycosylation of recombinant proteins with a high degree of terminal α2-3 sialylation even under high expression conditions. Some human tissue-specific terminal carbohydrate motifs are not synthesized by these cells since they lack the proper sugar-transferring enzymes (e.g. α1-3/4 fucosyltransferases, α2-6 sialyltransferases). Glycosylation engineering of these hosts by stable transfection with genes encoding terminal human glycosyltransferases allows to obtain products with tailored (human tissue-specific) glycosylation in high yields.

Using site-directed mutagenesis, unglycosylated polypeptides can be successfully converted in N- and/or O-glycoproteins by transferring glycosylation domains (consisting of 7-17 amino acids) from donor glycoproteins to different loop regions of acceptor proteins.

The genetic engineering of glycoproteins and of host cell lines are considered to provide a versatile tool to obtain therapeutic glyco-products with novel/improved in-vivo properties, e.g. by introduction of specific tissue-targeting signals by a rational design of terminal glycosylation motifs.

Keywords: **glycosylation engineering, human fucosyltransferases, human sialyltransferases, N- and O-glycosylation, host cell specificity, recombinant glycoprotein expression**

Introduction

The importance of the posttranslational modification of polypeptides with *N*- or *O*-linked oligosaccharides is well documented by their implication in numerous biological phenomena [1]. Consequently, it has already been recognized in the early eighties [2] that only mammalian host cells meet the criteria for an appropriate biotechnological development of recombinant glycotherapeutics to be used in humans. This has led to the attractive new research area of the biotechnology of mammalian cells as factories for medicinal glycoproteins.

Protein-linked oligosaccharides control the intracellular and tissue targeting of polypeptides, their half-life *in vivo* and their dynamic interaction with other proteins inside the cells or in body fluids. Carbohydrate structures of glycoproteins are typically polypeptide-specific and it has been shown that each individual glycosylation site of a glycoprotein may contain its own characteristic pattern of oligosaccharide chains [3]. Apart from the 3D-domain structure that governs its decoration with glycans, also the tissue or cell type that synthesizes a glycoprotein plays an important role in the phenomenon of microheterogeneity of protein glycans. This is a result of the regulated expression of a characteristic set of glycosidase and terminal glycosyltransferase genes which is different in the various cells/tissues of an organism [4] and may also vary with the physiological conditions of an organism or the differentiation state of cells. For example, human transferrin secreted from liver cells into the blood stream contains oligosaccharides usually found on serum glycoproteins, mostly afuco diantennary oligosaccharides with terminal α2,6-linked NeuAc, whereas the same protein isolated from human cerebrospinal fluid carries asialo and asialo-agalacto diantennary forms of proximally fucosy-

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lated chains with bisecting GlcNAc typical for glycoproteins synthesized in brain tissues [5–7] due to the different expression levels of the pertinent terminal glycosyltransferases.

Concepts for the biotechnological production of recombinant glycoprotein therapeutics or recombinant retrovirus vectors and *ex vivo* expansion of human primary cells for medicinal treatment must take into consideration different interactions of differently glycosylated cell/virus surface glycoconjugates or soluble glycotherapeutics with cellular receptors and subsequent altered modulation of intracellular signalling cascades. While during the past 12 years much work has been published on the structural characterization of recombinant glycoproteins expressed from various mammalian and nonmammalian expression systems, a great deal of efforts is presently going into attempts to improve recombinant host cell lines, and here especially mammalian cells, for the manufacturing of glycoprotein pharmaceuticals and retrovirus vectors with novel *in vivo* properties. It seems promising to explore the advantages of new generations of products with improved *in vivo* stability and carbohydrate-based tissue-targetable addressing signals. For this, the host cell lines must be improved by genetic engineering with newly introduced glycosyltransferases. The transferases must be stably directed into the proper subcellular compartment for their efficient function in the glycosylation pathway of the host. Here we are reporting on some of these aspects of the work from our laboratory at the German Center for Biotechnology (GBF) during the past 10 years.

I. Recombinant expression of human therapeutic glycoproteins

Mammalian host cells

Recombinant mammalian host cells cultured in large bioreactor systems are currently used to generate human glycoprotein pharmaceuticals which can be obtained from natural sources in only minute quantities. Amongst the first recombinant pharmaceutical glycoproteins produced from mammalian host cells were the secretory polypeptides interleukin-2 (IL-2), interferon-β (IFN-β and interferon-γ (IFN-γ) [8–10]. Over the past 12 years the literature reporting on the glycosylation analysis of recombinant glycoproteins from different hosts has accumulated tremendously. In most of these studies Chinese hamster ovary (CHO) and baby hamster kidney cells (BHK-21) have been used as expression systems and most of our present knowledge about the culture conditions that can affect the fine structural characteristics of recombinant glycoproteins produced in large scale processes has been obtained from studies with these two hosts cells [11–14]. In our hands, a constitutively secreted glycoprotein expressed at a level of 0.1 μg/ml from BHK-21 or CHO cells has the same carbohydrate structure as has the protein expressed at a 200-fold higher level, and as a general rule, it appears that the glycosylation machinery of the host cell itself is not a bottleneck for an efficient posttranslational modification of a polypeptide with carbohydrates. However, problems might eventually be encountered when a recombinantly expressed protein has an abnormal half-life in different cellular subcompartments of the host. It should be noted that in rare cases the selection procedures used for the isolation of transfected high expression cell clones may lead to the detection of a variant cell clone with aberrant glycosylation capacity, as has been recognized in our laboratory with a BHK-21 cell line that showed a complete loss of carrying out complex-type glycosylation of a recombinantly expressed protein [15].

As has become clear from the work of others and our own investigations, CHO and BHK-21 cells show basically the same characteristics for the glycosylation of recombinant *N*- or *O*-glycoproteins. The structural features detected in recombinant glycoproteins expressed from murine and hamster cell lines are summarized in Table 1. In principle, the antennarity and the LacNAc content of *N*-linked oligosaccharides of a given recombinant glycoprotein expressed in CHO, BHK-21 or the murine cell lines will be the same and this is also true for characteristics of

Table 1. Structural features of N-linked oligosaccharides from recombinant glycoproteins expressed in mammalian host cells. Data are based on structural analysis of the recombinant human glycoproteins IFN-β, Epo, AT III, IL-6, tissue-plasminogen activator and β-TP as well as recombinant humanized antibodies, soluble receptor proteins and *N*-glycosylation mutants of human IL-2.

	host cell line			
carbohydrate structure	*CHO*	*BHK-21*	*C127*	*Ltk⁻*
proximal fucose	+	+	+	+
Fuc(α1-2)Gal-R*	+	+	?	?
α2,6-NeuAc	–	–	+	+
α2,3-NeuAc	+	+	+	+
NeuAc(α2-8)NeuAcα2-3-R	+	+	–	+
NeuGly*	+	+/–	+	+
tri/tetra-antennarity	+	+	+	+
Gal(β1-4)GlcNAc repeats	+	+	+	+
Gal(β1-3)GlcNAc-R	–	+	–	–
sulfated glycans	+	+	+	+
Gal(α1-3)Gal	+	–	+	+
branched repeats	?	–	+	–
mannose 6-phosphate*	+	+	?	?
bisecting GlcNAc	–	–	+	+
GalNAc(β1-4)GlcNAc	–	+**	–	–

*detectable only in trace amounts
**detected in large amounts in the BHK-21A variant cell line [16,17]

the oligosaccharide pattern at individual glycosylation sites. However, in view of the pronounced higher microheterogeneity of terminal carbohydrate motifs in recombinant products obtained from the murine host cells (α2,3- vs. α2,6-NeuAc, NeuGly, Gal(α1→3)Gal, Gal(β1→3)GlcNAc-R, sulfated structures and branched repeats in L*tk*$^-$ and C127 cells), the two hamster cell lines seem to provide a more favourable expression host cell system when low glycoform heterogeneity is required.

It should be emphasized that in most publications on carbohydrate structures of recombinant glycoproteins the work has been performed with purified glycoprotein preparations destined for pharmaceutical use. Consequently, these preparations represent a subfraction of the total product secreted by the host cell and they are enriched in glycoforms which are believed to be most effective for *in vivo* application in humans. One such example is recombinant human erythropoietin (EPO) from BHK-21 or CHO cells where only a highly sialylated subfraction (based on the isoelectric focussing pattern) of the total recombinant glycoprotein hormone that is secreted by the producer cells is manufactured for medical treatment. This subfraction represents only about 20–25% of the total EPO secreted from the host cell lines. Therefore, for a complete description of the glycosylation characteristics of any host cell, it is indispensible to purify the product quantitatively, *e.g.*, by immunoaffinity chromatography using polypeptide-specific antibodies that guarantee >90% final yield.

Significant advances in the sensitivity of carbohydrate structural analysis has been achieved during the past three years. Especially in mass spectrometry (on-line ESI-MS, nanospray tandem mass spectrometry (ESI-MS/MS) and improved MALDI/TOF techniques), very sensitive instrumentation for glycosylation analysis has been made available to a broader group of research units, and thus has led to a broader use of complementary tools by academic researchers and in industrial laboratories. This is of outstanding importance in the area of glycobiology and glycotechnology where the combined methods of molecular biology, protein biochemistry, cell biology and analytical know-how are required to understand in detail the basic mechanisms and the role of modification of proteins and lipids with carbohydrate in both, health and different states of disease.

Production of secretory glycoproteins in insect cells using the recombinant baculovirus expression system

Some 10 years ago it has been proposed to use insect cells infected with recombinant baculoviruses for production of large amounts of recombinant glycoproteins. However, it has become clear that the insect expression system has its limitations for the production of mammalian-type modified glycotherapeutics. We found that secretory glycoproteins that contain complex-type *N*-glycans when expressed in mammalian host cell lines are modified only with the short oligomannosidic Man(α1→6)Man(β1→4)GlcNAc(β1→4)[Fuc(α1→6)]GlcNAc and Man(α→3)[Man(α→6)]Man(β1→4) GlcNAc(β1→4)[Fuc(α1→6)]GlcNAc *N*-glycans when expressed from Sf21 or Sf9 (*Spodoptera frugiperda*) cells [18]. *N*-glycan structures most similar to those synthesized in Sf9 or Sf21 cells were also detected in the products secreted from SPC-Bm36 (*Bombyx mori*) cells (see Table 2). SPC-Bm36 cells produce *N*-glycosylated proteins with higher amounts of dimannosyl- over trimannosyl-oligosaccharides and only 60% α1,6-fucosylation of the proximal GlcNAc. A model glycoprotein with a potential *O*-glycosylation motif [18] expressed from SPC-Bm36 cells was found to be unglycosylated, modified with GalNAc or with Gal(β1→3)GalNAc in a ratio of 1:3:5 that is different in the same protein when synthesized from Sf21 cells, where a ratio of 1:4:4 was detected [18]. Therefore, it appears that SPC-Bm36 cells generally underglycosylate *N*- and *O*-glycoproteins.

N-glycan structures of glycoproteins expressed from BTI-Tn-5B1-4, "High Five" (*Trichoplusia ni*) cells are essentially the same as those from Sf21 cells and are present in a similar ratio. However, a considerable proportion of the oligosaccharides was found to be *difucosylated,* containing an additional fucose in α1,3-linkage to the proximal GlcNAc. The enzymatic activity involved in the biosynthesis of this structural motif has also been described for a *Mamestra brassica* cell line, IZD Mb0503 [19]. Surprisingly, the analysis of a *N*-glycosylated protein variant expressed in BTI-EaA (*Estigmene acrea*) cells revealed the presence of fucosylated trimannosyl-oligosaccharides containing 1,2, or small amounts of even 3 terminal GlcNAc-residues as detected by methylation analysis and ESI-MS/MS of the pertinent tryptic glycopeptide [20]. However, no indication for galactosylated oligosaccharides was detected by the complementary analytical techniques applied in our laboratory. Such complex-type *N*-glycan structures were not detected on recombinant glycoproteins expressed in any other baculovirus-infected insect cell line shown in Table 2. These data clearly confirm our previous results [18] and those of others [21,22] that insect cells lines are incapable of synthesizing sialylated lactosamine complex-type *N*-glycans or sialylated core 1 *O*-glycans and therefore are not suitable for the production of recombinant pharmaceutical glycoproteins for clinical use.

An further disadvantage of the insect cell expression system is that recombinant baculovirus vector-driven high expression of proteins is run as a batch-culture process and cells die after infection within the productive phase. Only about 20% of the total recombinant protein synthesized by the host cells is secreted into the supernatant. The remainder is found denatured as inclusion body-like aggregates

Table 2. Glycosylation characteristics of recombinant glycoproteins secreted from 6 different lepidopteran cell lines. Data were obtained by detailed carbohydrate analysis of human β-TP, IFN-β and different human IL-2 *N*-glycosylation variants secreted from baculovirus-infected cells. Cell cultures were run in tissue culture flasks and in bioreactors, and the products were analysed from harvests after 2 days, 4 days, and 7 days post infection. M = mannose, Gn = *N*-acetylglucosamine, F = fucose

	N-glycan structures of secreted recombinant glycoproteins									
Insect cell line	M \ M—Gn—Gn	M \ M—Gn—Gn / M	M F \ |6 M—Gn—Gn	M F \ |6 M—Gn—Gn / M	M \ M F / \ |6 M M—Gn—Gn / M	M F \ |6 M—Gn—Gn |3 F	M F \ |6 M—Gn—Gn / |3 M F	M F \ |6 M—Gn—Gn / Gn—M	Gn—M F \ |6 M—Gn—Gn / Gn—M	Gn \ Gn—M F \ |6 M—Gn—Gn / Gn—M
Spodoptera frugiperda (Sf21)	+	+	+++	+++	+	–	–	–	–	–
Spodoptera) frugiperda (Sf9	+	+	+++	+++	+	–	–	–	–	–
Trichoplusia ni (BTI Tn5B1-4, "High-Five")	+	+	++	++	–	++	++	–	–	–
Bombyx mori (SPC-Bm36)	+++	+	+++	+	–	–	–	–	–	–
Mamestra brassicae (IZD Mb0503)	–	–	++	++	–	+++	++	–	–	–
Estigmene acrea (BTI-EaA)	–	–	++	++	–	–	–	++	++	+

inside the cell and is partially released into the medium at later states of infection from dying cells [23]. Nevertheless, the baculovirus expression system is the preferred system when the production of several 100 mg of a protein is required for research purposes within a short time and a mammalian/human-type of glycosylation is not of primary importance.

II. Genetic engineering of new glycoproteins and their recombinant expression in animal cells

Engineering of proteins with new glycosylation properties

The successful modification of a polypeptide with newly introduced *N*- or *O*-glycosylation properties may increase its solubility or influence its *in vivo* biological properties (e.g., activity, antigenicity, rate of clearance). In other cases, the simple deletion of a glycosylation site with pronounced carbohydrate microheterogeneity might also be of advantage for a final clinical application of the product. It is now known from the 3D-structures of many glycoproteins that *N*- or *O*-glycosylation motifs are mostly found in loop-regions of polypeptides. According to our experience, a single amino acid exchange creating a new potential consensus tripeptide Asn-Xxx-Ser/Thr is often not sufficient for *N*-glycosylation to occur, even when present in loop regions. The concept of polypeptide-specific and glycosylation site-specific modification of proteins with carbohydrates has led us to investigate by using site-directed mutagenesis the introduction of individual *glycosylation domains* from donor glycoproteins with known glycosylation characteristics into suitable locations of model acceptor proteins (IL-2, IFN-β. This approach aimed at the definition of short peptide domains that should result in predictable oligosaccharide structures when the constructs are expressed from a given host cell. The insertion of short (8–15 residues) peptide sequences containing an Asn-Xxx-Thr/Ser sequence has been proved to be successful, and, most importantly, was found not to severely affect the overall 3D-structure as shown for the biological activity of the resulting human IL-2 and IFN-β variants [23,24]. We have analyzed the carbohydrate structure of the different resulting chimers after expression from BHK-21 cells as shown in Figure 1. The single *N*-glycosylation site of human IFN-β contains preponderantly diantennary complex-type oligosaccharide chains when expressed from CHO or BHK-21 cells [9,25], as is the case for all four *N*-glycosylation sites of human antithrombin III (AT III) [26]. The three *N*-glycosylation sites of human EPO from the same host cells contain preponderantly tetraantennary chains with 1-3 *N*-acetyllactosamine repeats [27–30], with Asn_{86} (site III) bearing the most homogenous oligosaccharide population. As mentioned above, the introduction of a new *N-glycosylation site* into the IL-2 polypeptide by substitution of $Thr_3 \rightarrow Asn$ ($APNSSSTKKT_{10}$..) does not result in any modification with *N*-glycans when the construct is expressed from BHK-21 or L*tk*$^-$ cells [24]. However, the transfer of the human IFN-β *N-glycosylation domain* SSSTGWNETIV(GG) to the N- or C-terminus of IL-2 yielded proper *N*-glycosylation with diantennary complex-type chains [31,32], as is the case for wild-type IFN-β from these hosts [9,25].

Similarly, the peptide comprising the *N*-glycosylation domain III of human AT III was found to be modified with diantennary *N*-glycans when inserted at position 80 within the loop region between helices B′ and C of human IL-2 as is depicted in Figure 1. However, the introduction of *N*-glycosylation domain III of human EPO at the same location resulted in oligosaccharides with significantly higher antennarity. These investigations indicate that *N*-glycosylation domains can successfully be transferred from one protein to a loop region or the N- or C-terminus of another protein. Our data allow for the conclusion that in several cases the characteristic antennarity of the donor *N*-glycosylation domain is preserved when inserted into the newly constructed mutant glycoprotein [11,23,24,31,32].

Engineering of O-glycosylated proteins

All mammalian cell lines frequently used for recombinant protein expression (*e.g.* the cell lines in Table 1) modify *O*-glycosylation sites with preponderantly core 1 *O*-glycans containing one or two NeuAc [27,29,33]. In secretory glycoproteins like human IL-2 or EPO, which are *O*-glycosylated at a single hydroxyamino acid, all recombinant host cells recognize specifically the same Ser or Thr that is modified in the natural protein even when it is part of a hydroxyamino acid cluster [11,24], as is the case for human IL-2 where specifically Thr_3 within the N-terminal sequence H_2N-$APT_3SSSTKKT_{10}$ . . . is modified by NeuAc(α2→3)Gal(β1→3)[NeuAc(α2→6)]$_{0-1}$GalNAc chains [33]. As is summarized in Table 3, mutant IL-2 proteins with substitution of $Thr_3 \rightarrow Ser$ or containing Thr at position 5 are not *O*-glycosylated [24]. The deletion of Thr_3 or its substitution with Ala or Ser abolishes *O*-glycosylation completely, whereas the exchange of Ser_4 or Ser_6 to Thr results in detectable *O*-glycosylation. The peptide sequences APTPP, APTAPPT (present in human plasminogen) or the artificial sequence APTPPP can be used to introduce novel *O*-glycosylation sites into human IL-2 or human IFN-β at different positions of the polypeptide chain [11,18,24,32], and the resulting proteins are efficiently *O*-glycosylated in BHK-21 cells and L*tk*$^-$ cells, as is indicated also in Figure 1. Thus, these sequence motifs can be considered to constitute general transferable *O*-glycosylation recognition domains when introduced into loop regions of polypeptides although no consensus sequence for the attachment of *O*-glycans to polypeptides has yet been identified.

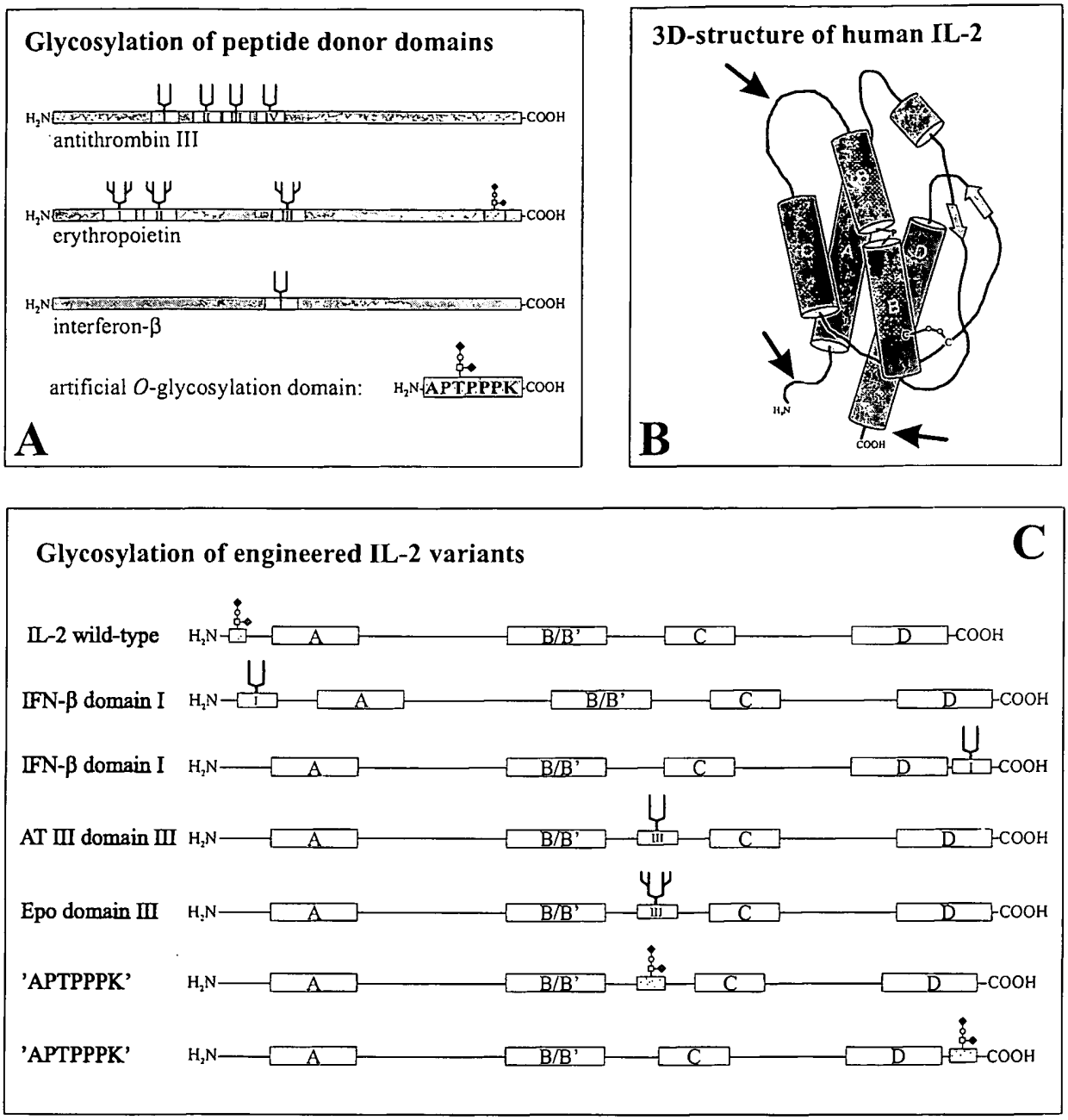

Figure 1. Genetic engineering of glycoproteins with defined glycosylation characteristics: Insertion of *N*- and *O*-glycosylation domains into human IL-2. *panel A*, glycosylation domain donor glycoproteins with known glycosylation characteristic; *panel B*, 3D-structural model of human IL-2 based on NMR data; arrows indicate acceptor sites used for insertion of glycosylation domains; *panel C*, glycosylation acceptor characteristics of newly introduced domains as identified by analysis of the resulting IL-2 variants. Bars A, B/B', C and D indicate helical domains of human IL-2 as shown in Figure 1*B*

Table 3. Mutation analysis of the *O*-glycosylation acceptor properties of the N-terminus of human IL-2. Data were corroborated by immunoprecipitation of stably (BHK-21) as well as transiently (L*tk*⁻) transfected mammalian host cells and N-terminal sequencing of the purified proteins.

N-terminal sequence	*O-glycan attached at position*
APTSSSTKKT . . .	3
APASSSTKKT . . .	no
APSSSSTKKT . . .	no
APSSSTKKT . . .	no
APTISSTKKT . . .	3↑
APTSISTKKT . . .	3↓
APTSSITKKT . . .	3↑
APNSSSTKKT . . .	no
APSISSTKKT . . .	no
APSSISTKKT . . .	no
APSSSITKKT . . .	no
APTAPPTKKT . . .	3↑, 7
APTPPSTKKT . . .	3↑
APTPPPTKKT . . .	3↑

III. Construction of host cell lines with novel glycosylation characteristics

In vivo specificity of glycosyltransferases

In vitro assays of glycosyltransferases with small acceptor substrates may yield some preliminary information about acceptor substrates properties recognized by the enzymes and are indispensible for the evaluation of glycosyltransferase levels in cells/tissues and the control of enzyme purification. A final description and comparison of the *in vivo* specificity of the individual glycosyltransferases, however, can only be achieved by structural analysis of the cellular product(s). As shown in Figure 2, we suggest the recombinant expression of the full length form of human glycosyltransferases along with a suitable reporter glycoprotein (here human β-TP) at a constant expression level in a heterologous mammalian host cell line that is devoid of the pertinent enzyme activity. This is considered to represent a valuable model and should enable the comparison of the *in vivo* specificities of different members of a glycosyltransferase family [16,17,34,35] and allow the selection of the optimal enzyme suitable for the glycosylation engineering of host cell lines for the production of a new generation of glycotherapeutics with defined altered glycosylation characteristics. Basic information can be expected from such

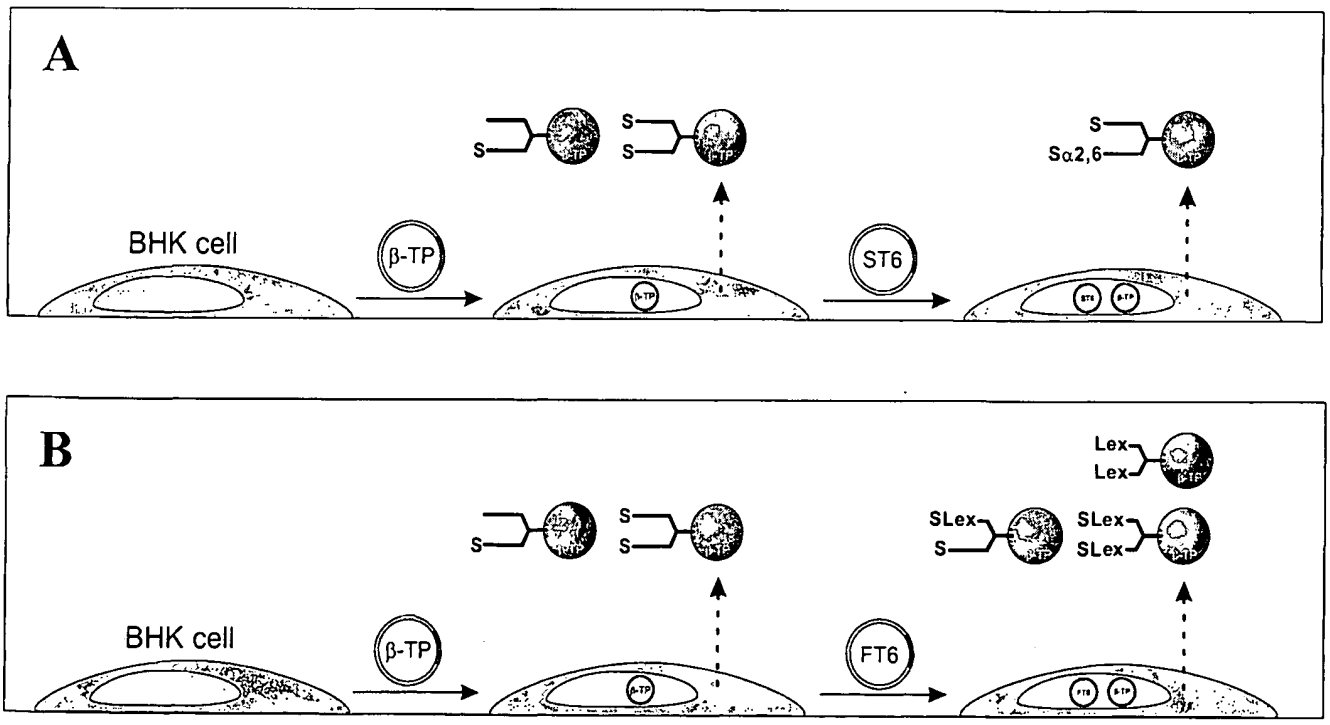

Figure 2. Genetic engineering of new BHK host cells by transfection with human glycosyltransferase genes. In order to generate expression of a reporter glycoprotein, BHK-21 cells were transfected with a plasmid encoding human β-TP. The recombinant β-TP secreted from such cell lines is glycosylated host cell-type-specifically with α2,3-di- or monosialo diantennary complex-type *N*-glycans (with proximal fucose) as indicated by "S" attached to the structural symbols. *panel A*, cotransfection with the human ST6Gal I gene leads to secretion of β-TP containing α2,6-linked NeuAc; *panel B*, cotransfection with human α1,3-fucosyltransferase VI (FT6) results in β-TP modified with sLex- or Lex-containing oligosaccharides

studies concerning the intracellular organization of the protein glycosylation machinery and the temporal and spatial distribution of the transferases in the *in vivo* biosynthetic compartments. Prerequisites for such an *in vivo* assay system are:

i. Reproducible transfection procedures using high expression vectors and rapid selection/isolation of stably transfected cells
ii A constant level of acceptor substrate expression (reporter glycoconjugate) by the host cells
iii. A defined expression level of the recombinant enzymes (20- to 50-fold higher expression levels of the wild-type forms are achieved in transfected cells when compared to the levels in primary cells/tissues)
iv. A simple, fast and quantitative purification procedure for the product (preferably secreted into the medium)
v. Application of fast and sensitive carbohydrate structural analytical micromethods (MS and MS/MS-techniques, HPAE-PAD)

It should be emphasized that the 3D-structure of the glycoprotein substrate and thus the accessibility of its oligosaccharide moieties under the intracellular environmental conditions is also of importance. In addition, it is conceivable that cell surface membrane glycoproteins might be recognized differently than are obligate secretory glycoproteins. However, for a given model glycoprotein with defined structural characteristics, the above approach by analysis of a reporter glycoconjugate from stably transfected cells should yield precise information on the *in vivo* substrate specificity of the individual members of a family of enzymes acting on the same precursor substrate. Transient expression experiments are of limited value, since cell damage and cell leakage resulting from the transfection procedures is considered to lead to artefacts.

Engineering of cells by stable transfection with human α1,3/4-fucosyltransferases genes

Analysis of the in vivo acceptor substrate specificity of fucosyltransferases by glycosylation analysis of coexpressed recombinant human β-trace protein.

The α1,3/4-fucosyltransferases III-VII [36–41] add fucose to the GlcNAc residue in sialylated or unsialylated Gal(β1→3)GlcNAc-R or Gal(β1→4)GlcNAc-R type structures of glycoconjugates. They are thus involved in the regulation of the synthesis of the Lewis X (Lex) and sialyl Lewis X (sLex) type ligands that are involved in inflammation-induced adhesion of neutrophils, monocytes, T cells and platelets to selectins [42–45]. Fucosylated glycoconjugates play also a central role in other important biological phenomena like differentiation and tumorigenesis, and elevated levels of peripherally fucosylated serum glycoproteins have been detected in humans associated with inflammatory processes [46,47].

Many natural human tissues/cells express more than one fucosyltransferase at the same time and therefore it is difficult to obtain homogenous enzyme preparations from natural tissues or body fluids for the unequivocal assessment of the specificity of the individual enzymes. The cloning of the α1,3/4-fucosyltransferases III-VII (FT3-FT7) and their expression in recombinant form has provided a tool to isolate pure enzyme preparations for studying their substrate specificity *in vitro*. However, several questions concerning the implication of each of the individual fucosyltransferases in the generation of selectin ligands are still not resolved.

Mammalian glycosyltransferases are Golgi-resident type II transmembrane proteins, and according to current opinion, their transmembrane region is responsible for the retention of the enzymes in the proper Golgi compartment. Many transferases contain *N*-glycosylation sites in their stem region and/or their catalytic domain; however, no information is available if, or to what extent, *N*-glycosylation is involved in the *in vivo* activity or specificity of glycosyltransferases. A number of publications have appeared that describe the recombinant expression of human fucosyltransferases [48–52] mostly as soluble forms lacking the cytoplasmic, the transmembrane and some part of the stem region. In several cases, recombinant chimers containing N-terminally fused polypeptide fragments (*e.g.*, of protein A) have been constructed to facilitate recombinant enzyme purification. According to the data published so far, FT7 has been reported to fucosylate exclusively α2,3-sialylated *N*-acetyllactosamine-type structures *in vitro* and is inactive with neutral acceptors [41,51,52]. FT4 acts almost exclusively on unsialylated Gal(β1→4)GlcNAc-R (type **II**) structures [48,53], whereas *in vitro*, FT5 and FT6 have been reported to act on both, α2,3-sialylated as well as unsialylated type **II** acceptors [48,49,54]. FT3 has been reported to mainly transfer Fuc in α1,4-linkage onto GlcNAc in type **I** chains [34,48,54]. Activity with type **I** acceptors has also been found for human FT5 [48], while FT4, FT6 and FT7 are not active with Gal(β1→3)GlcNAc-R substrates [41,48,54].

Human β-TP is a 168 amino acid protein which contains two *N*-glycosylation sites that are occupied with almost exclusively diantennary complex-type chains [5,16,35,55]. Similar to human transferrin described above, β-TP isolated from human cerebrospinal fluid exhibits "brain-type" glycosylation characteristics, *i.e.*, mainly truncated asialo chains, bisecting GlcNAc, complete proximal and some peripheral fucosylation besides small amounts of α2,3/6-sialylated *N*-glycans [5,7]. Recombinant human β-TP expressed from wild-type BHK-21B cells is also modified with almost exclusively diantennary oligosaccharides at each of its two *N*-glycosylation sites, however, as shown in Figure 3, the oligosaccharide pattern here is very homogenous, the structures contain either two or one α2,3-linked NeuAc and only small amounts of asialo chains are present [16,35].

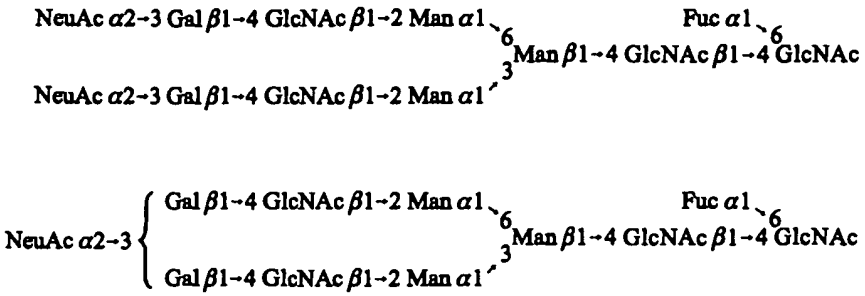

Figure 3. *N*-glycan structures of recombinant human β-TP secreted from *wild-type BHK-21* cells contain exclusively α2,3-linked NeuAc.

Coexpression of β-TP as a reporter glycoprotein from cells transfected with a human α1,3/4-fucosyltransferase therefore should yield oligosaccharides with Lewis X or sialyl Lewis X motifs or mixtures of the two motifs depending on the *in vivo* specificity of the transfected fucosyltransferase gene. In total 12 different diantennary *N*-linked oligosaccharides can be expected in β-TP from BHK-21 cells expressing human FT6 (four each of asialo, mono- and disialo chains with no, one or two peripheral fucose residues, respectively).

Human FT6 synthesizes preponderantly α1,3-difucosylated structures on diantennary chains *in vivo*

When incubated *in vitro* with soluble FT6 in the presence of GDP-Fuc, we found efficient fucosylation of β-TP, with a roughly 50% modification of the α2,3-monosialylated oligosaccharide with one α1,3-linked Fuc, and with 33% and 6% modification of the α2,3-disialylated oligosaccharides with one or two α1,3-linked Fuc residues, respectively [35]. This result obtained for the complex type *N*-glycans is in agreement with published reports for the *in vitro* specificity of recombinant human FT6, which indicate that the enzyme can form Lex as well as sLex motifs with small type **II** oligosaccharides [49,54]. When β-TP is coexpressed from BHK-21 cells together with human FT6 (see Table 4), about 50% of all *N*-glycans contain α1,3-linked Fuc (*cf.* Fig. 4*A*). However, the sialylation degree of the *N*-glycans is significantly lower when compared to *N*-glycans of β-TP from wild-type BHK cells (Fig. 4*B*), and also in contrast to the *in vitro* modified β-TP, most of the oligosaccharides are found to be modified *in vivo* with *two* peripheral Fuc (see

Table 4. Fucosyltransferase activities of stably transfected BHK-21 cell lines. Measurements were performed using the substrate GDP-[^{14}C]Fuc and Gal(β1→4)GlcNAc—O—$(CH_2)_8$—$COOCH_3$ as an acceptor. A dash indicates incorporation of radioactivity at background levels. *FT3 activity was detected by using the type **I** Gal(β1→3)GlcNAc-O-$(CH_2)_8$-$COOCH_3$ acceptor; **FT7 was determined with native bovine fetuin. *In vitro* activity values for FT5 and FT7 are very low; however, from standard transfection procedures used, and in view of the *in vivo* fucosylation efficiency (see Fig. 5), an expression value similar to those detected for the other fucosyltransferases is assumed.

Cell line	*Total activity (cells + culture medium) $\mu U \times 10^{-6}$ cells × 48 h^{-1}*	*% of total activity accumulated in the culture medium after 48 hours*
BHK-21B (wild-type)	—	—
FT3	—	—
FT3*	100	78%
FT4	75	27%
FT5	1	0%
FT6 (BHK21-B)	170	74%
FT6 (BHK-21A)	210	88%
FT6 (CHO DHFR-)	370	81%
s-FT6(I)	175	92%
s-FT6(II)	2970	91%
BT-FT6	9400	96%
FT7	—	—
FT7**	3	0%

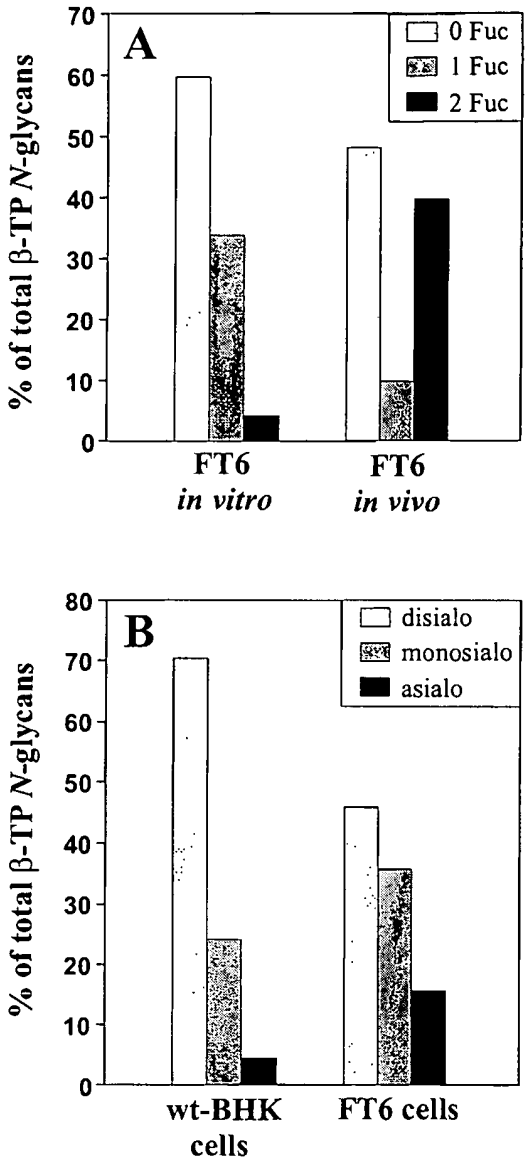

Figure 4. Comparison of β-TP oligosaccharides after *in vitro* or *in vivo* modification with human FT6. For *in vitro* fucosylation, purified β-TP expressed from BHK-21 cells was incubated with recombinant soluble human FT6 (s-FT6) in the presence of GDP-Fuc. *In vivo* data were obtained following coexpression of β-TP and full-length FT6 genes in BHK-21 cells (*cf.*, Fig. 2). *panel A*, percentage of nonfucosylated, α1,3-mono- and α1,3-difucosylated *N*-glycans; *panel B*, percentage of disialo, monosialo and asialo diantennary chains in β-TP *N*-glycans

Table 5. In vivo fucosylation characteristics of human α1,3/4-fucosyltransferases. Values represent the percentage of all diantennary oligosaccharide forms comprising >90% of total N-glycans isolated from β-TP expressed in each individual FT-transfected BHK-21 cell line. Structural analysis was performed by HPAE-PAD mapping, MALDI/TOF-MS, ESI-MS/MS and methylation analysis. The two isomeric monosialo/monofucosylated structures from FT3, FT5 and FT6 cells were not resolved.

	β-TP N-glycan structures (%)										
BHK cell line	S S	S		SLex SLex	SLex S	SLex Lex	SLex	Lex S	Lex Lex	Lex
wild-type	75	20	5	—	—	—	—	—	—	—
FT3	59	20	2	3	13	—	— 3	—	—	—
FT4	40	9	—	—	5	4	—	21	17	3
FT5	46	18	2	8	12	5	— 7	—	2	—
FT6	29	16	3	13	5	15	— 5	—	13	—
FT7	43	22	4	11	11	—	9	—	—	—
s-FT6(I)	59	35	6	—	—	—	—	—	—	—
BT-FT6	55	17	2	5	7	6	— 3	—	4	1

also Table 5). The ratio of sLex:Lex antennae in the total *N*-glycan mixture is 1.1:1. The lower sialylation degree of β-TP from FT6 cells compared to wild-type BHK-21 cells can be explained by an *in vivo* competition of the recombinantly expressed FT6 with the endogenous α2,3-ST(s), ST3Gal III and/or ST3Gal IV, for the common asialo oligosaccharide substrate. This phenomenon has been observed previously for products from recombinant host cell lines transfected with α1,3-galactosyltransferase [56] or FT4 [57]. The α2,3-STs have been reported to be unable to sialylate Gal(β1→4)[Fuc(α1→3)]GlcNAc-R motifs [41,50,57]. Obviously, as is the case for *in vitro* incubation conditions, FT6 can act also *in vivo* on both, α2,3-sialylated as well as unsialylated *N*-linked oligosaccharides. To our knowledge, this is the first publication describing quantitatively and in detail the *in vivo* substrate specificity of a recombinant fucosyltransferase expressed in a stably transfected heterologous host cell line [35].

All human α1,3/4-fucosyltransferases synthesize sLex structures *in vivo*

The differences of the *in vitro* and *in vivo* results obtained for FT6 led us to compare the *in vivo* properties of all human α1,3/4-fucosyltransferases. For this, we have also constructed stable BHK-21 cell lines (see Table 4) expressing human FT3, FT4, FT5 or FT7 together with human β-TP as a reporter glycoprotein, and for each individual cell line, β-TP was purified from the culture supernatant and subjected to oligosacharide structural analysis using MALDI/TOF-MS, ESI-MS/MS and HPAE-PAD mapping. About 30–50% of the *N*-linked oligosaccharides of β-TP secreted from the new cell lines were α1,3-fucosylated except for the FT3 cell line in which case only 19% of the structures were fucosylated. A comparison of all β-TP *N*-glycan structures formed by the α1,3/4-FT-transfected cells is presented in Table 5 and gives an overview of the *in vivo* substrate specificity of the five known human α1,3/4-FTs with protein-bound complex-type *N*-linked oligosaccharides [35].

FT7 cells (*i.e.*, BHK-21 cells coexpressing FT7 and β-TP) exclusively synthesize sLex structures. We have confirmed this also for the monosialylated oligosaccharide fraction that did not contain any α1,3-difucosylated structure. The single Fuc was exclusively present as the sLex and *not* as the Lex motif (determined by ESI-MS/MS, see. ref. [35]), and the small amount of asialo oligosaccharides did not contain any Lex epitopes. This *in vivo* specificity with complex-type *N*-glycans is in agreement with recent work published on the *in vitro* activity of the enzyme with small oligosaccharide substrates [41,50,51].

The vast majority of the peripherally fucosylated product in β-TP oligosaccharides from **FT4 cells** were found to contain the Lex motif which result is compatible with published data on the *in vitro* activity of FT4 with low molecular weight compounds. From the fragmentation pattern of reduced and permethylated chains using ESI-MS/MS, we could show that the monosialylated α1,3-monofucosylated *N*-glycan contains preponderantly the Lex motif [35]. However, a significant amount (11%) of mono-sLex was also observed in the disialo oligosaccharide fraction which contradicts published data on the *in vivo* specificity of the enzyme as measured by E-selectin binding studies [41,53,58,59]. However, no α1,3-difucosylated disialo structure was observed, supporting the view of the preferential action of FT4 on nonsialylated Gal(β1→4)GlcNAc-R structures.

FT5 cells secrete β-TP with oligosaccharides modified preponderantly with the sLex motif, but also Lex-containing structures are formed. They were detected as α1,3-difu-

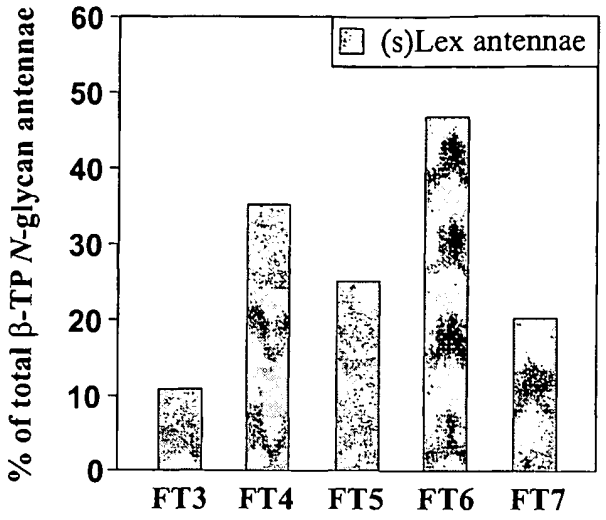

Figure 5. Comparison of fucosylated oligosaccharide antennae of β-TP secreted from cell lines cotransfected with human fucosyltransferases III–VII (compare also Table 4)

cosylated asialo and α1,3-difucosylated monosialo oligosaccharides. In contrast to the situation found for the monosialo-monofuco oligosaccharides from FT7 and FT4 cells, the α1,3-monofucosylated monosialo oligosaccharides from FT5 cells were found to consist of a mixture of Lex and sLex containing structures.

In a previous study [34], we have reported that human **FT3** from BHK-21 cells does not fucosylate type **II** *N*-acetyllactosamine structures in several glycoproteins when incubated *in vitro*. Furthermore, we could clearly demonstrate that, with bovine fetuin as a substrate, only the triantennary oligosaccharide containing one type **I** branch (Gal(β1→3)GlcNAc) is modified with α1,4-linked Fuc *in vitro*, although an 8-fold higher type **II** acceptor concentration (Gal(β1→4)GlcNAc-R branches) was present during the experiment [34]. Similarly, we could show that no sLex or Lex *in vitro* activity was present in extracts of FT3 cells when using low molecular weight type **II** oligosaccharide acceptors (see also Table 4). These findings have been further confirmed in a recent publication from our group [54] describing the failure to *in vitro* fucosylate diantennary type **II** oligosaccharides with large amounts of a purified recombinant FT3 preparation. For the *in vivo* activity of the Golgi form of the enzyme, almost no fucosylation of asialo branches was observed in β-TP oligosaccharides from FT3-transfected cells. Using ESI-MS/MS and methylation analysis, we confirmed that only the sLex and no sLea structure was present in the oligosaccharides of coexpressed β-TP. Since only 11% of the total oligosaccharide antennae of β-TP from FT3 cells were modified with peripheral fucose compared to up to 50% of the total antennae in β-TP from cells transfected with FT4-FT7 (*cf.* Fig. 5), we conclude that FT3 preferentially acts *in vivo* as a type **I** chain-specific transferase which is in agreement with the *in vitro* data published previously [34,54].

The results of our *in vivo* specificity studies of recombinant human α1,3/4-FTs indicate that each of the enzymes exhibits a specific fucosylation characteristics with type **II** complex *N*-glycan chains on coexpressed human β-TP as is exemplified by the different sLex/Lex ratios: FT7 (only sLex) > FT3 (14:1) > FT5 (3:1) > FT6 (1.1:1) > FT4 (1:7) [35]. Furthermore, from the results obtained, recombinant human FT6 turns out to have a high *in vivo* preference to form α1,3-difucosylated structures with all three, asialo, mono- and disalo diantennary acceptor oligosaccharides. A similar high preference for the synthesis of α1,3-difucosylated diantennary glycans is only detected for FT4 with the asialo structures (compare Table 5). Apart from its strict specificity towards α2,3-sialylated antennae, FT7 appears to have very similar preference for both, α1,3-mono- and α1,3-di-Fuc-transfer onto *N*-linked oligosaccharides, whereas FT3 and FT5 predominantly attach a single peripheral Fuc residue to diantennary *N*-glycans.

Human FT6 requires Golgi membrane localization for its *in vivo* activity

In a previous publication we have reported that the wild-type Golgi form of human FT3 is intracellularly cleaved in stably transfected BHK-21 cells and the catalytically active fragment can be detected by *in vitro* assays and Western blotting in the cell supernatant [34]. Similar observations have been published for α2,6-sialyltransferase [60], β1,4-galactosyltransferase [61], α1,3-galactosyltransferase [62], polypeptide α1→O GalNAc-transferase [63], G_M2 synthase (β1,4-GalNAc-transferase) [64] and FT6 [65]. The enzymes responsible for this proteolytical cleavage have been proposed to be cathepsin-like proteases or serine proteases, respectively. We have found that recombinant human FT6 is secreted by two different BHK-21 cell lines and from CHO DHFR cells [35], as shown in Table 4. While FT7 and FT5 were found to be resistant to proteolysis in transfected BHK-21 cells, we have also observed secreted forms of FT4 in supernatants of cells transfected with wild-type human FT4. It is important to consider the proteolytically cleaved enzyme forms when describing the *in vivo* specificity of glycosyltransferases, since it is known that also from natural cells/tissues considerable amounts of soluble forms have been detected in the medium of cells or in body fluids in certain diseases [66,67].

We have addressed the question of a possible contribution to the *in vivo* activity of soluble forms by engineering of cells that express variants of human FT6 lacking the cytoplasmic and transmembrane domain and part of the stem region (s-FT6), as depicted in Figure 6. In addition, we have constructed a chimeric secretable protein (BT-FT6) by fu-

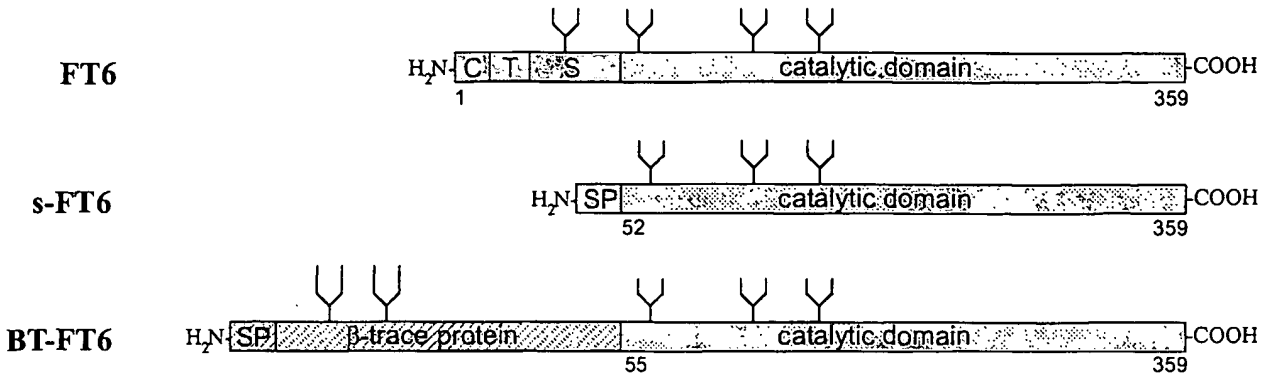

Figure 6. Schematic representation of the protein domain structures of wild-type human FT6 and soluble variants. C, T, S denote the *c*ytoplasmic, *t*ransmembrane and *s*tem regions; SP = signal peptide; the number of potential *N*-glycosylation sites are indicated by symbols

sion of the full-length human β-TP sequence to the N-terminus of the catalytic domain of human FT6 which resulted in a 70-fold overexpression of catalytic activity. Our results obtained with the coexpression of s-FT6 together with β-TP indicate that enzymes secreted along the secretory pathway can be considered to not contribute to the *in vivo* functional activity of the enzyme, since only after about 20-fold overexpression of s-FT6 (cell line s-FT6(II), compare Table 4), we were able to detect very small amounts of fucosylation of β-TP, and even 70-fold overexpressed BT-FT6 results in a low α1,3-fucosylation of β-TP, as shown in Table 5. Cho and Cummings [68] found by lectin binding that a recombinant, soluble α1,3-galactosyltransferase (lacking the transmembrane and cytoplasmic domain) is functionally active *in vivo* when expressed at slightly higher levels than the full-length form of the enzyme. The reason for this discrepancy is unknown; however, that truncated forms of glycosyltransferases in general do not contribute significantly to the *in vivo* specificity of the enzyme towards secreted glycoproteins is supported by our finding that a recombinant soluble form of human ST6Gal I does not modify co-secreted β-TP in BHK-21 cells. This finding is confirmed by a recent publication [69] where the authors describe that soluble forms of recombinant β1,4-GalNAc-transferase or ST6Gal I were not at all or significantly less efficient *in vivo* than their membrane-bound counterparts. It should be emphasized that in natural tissues or cells transferase expression levels are much lower than those that can be achieved by transfection of cells with corresponding plasmids when the gene is under the control of a strong promoter.

Interestingly, in addition to the very low fucosylation efficiency of the high enzyme activity expressing s-FT6(II) cell line in our studies, also the fucosylation pattern of β-TP oligosaccharides was different with a higher proportion of α1,3-monofucosylated structures observed over the α1,3-difucosylated oligosaccharides which are the major *N*-glycans synthesized by cells transfected with the full-length form of FT6 [35]. This then supports the view of the importance of the *c*ytoplasmic, *t*ransmembrane and *s*tem region (*CTS*-region) not only for the *in vivo* functional activity of glycosyltransferases, but also for their *in vivo* substrate specificities. In this context, it seems attractive to speculate that the CTS-region is also involved in the addressing of glycosyltransferases into different subcompartments of the biosynthetic glycosylation pathway of cells. The CTS polypeptide domains would be responsible for the targeting of the recombinantly expressed FT6 and FT4 to subcompartments where they can compete with the BHK cell endogenous ST3Gal III/IV for the same acceptor substrate since the sialylation state is lower for the *N*-glycans of β-TP secreted from the transfected cells (and as mentioned above, this indicates that ST3Gal III and ST3Gal IV do not recognize Lex motifs *in vivo*). The targeting properties of the FT6 CTS-region should result in an intracellular broader distribution of this enzyme functional activity and its overlapping with ST3Gal III/IV. FT4 should be targeted into an earlier Golgi subcompartment before the α2,3-STs modify the acceptor Gal(β1→4)GlcNAc-R substrate. Likewise, the CTS-region should direct FT7 and FT5 into a later functional compartment than the BHK cell endogenous

α2,3-STs which must provide the properly sialylated oligosaccharide precursor substrates.

Engineering of cells by stable transfection with human Gal(β1→4)GlcNAc-R α2,6- sialyltransferase

BHK-21 cells and CHO cells do not express *N*-glycan-specific α2,6-sialyltransferases (see Table 1). Therefore, glycoproteins with this typical human serum-type sialylation characteristic are not synthesized by these host cell lines. The stable transfection of cells with plasmids encoding human ST6Gal I [70] seems to be an attractive way to manipulate host cell lines for the production of this human serum-type carbohydrate structural motif. This experimental approach is justified by considering data published for the *in vitro* specificity of ST6Gal I as well as structural studies on natural glycoproteins. However, it has to be considered that the newly introduced enzyme competes with the host cell endogenous α2,3-STs (ST3Gal III/IV) for the same precursor substrate Gal(β1→4)GlcNAc-R as has been discussed above for human FT6 and FT4. In order to evaluate such a competition, we have stably transfected BHK-21B cells with a plasmid encoding the Golgi-resident form of human ST6Gal I and have coexpressed human β-TP as a secretory model glycoprotein.

As depicted in Figure 3, human β-TP from wild-type BHK cells contains simple diantennary chains which are α2,3-disialylated, monosialylated, or asialo in a ratio of 70:25:5 [16,35]. BHK-21B cells were stably transfected with plasmids encoding human β-TP and human ST6Gal I (*cf.* Fig. 2) and were used for the subsequent production of β-TP. Isolation of the secreted β-TP from cell supernatants was performed by a single step using immunoaffinity chromatography with a polypeptide-specific monoclonal antibody raised against β-TP [55]. Careful structural analysis of the *N*-linked oligosaccharides of the recombinant β-TP by complementary techniques (MALDI/TOF-MS, HPAE-PAD and NMR methods [16]) was performed and we found that monosialylated and disialylated glycans were present in a ratio of 1:5. As shown in Figure 7, 60% of the disialylated oligosaccharides contained both, α2,3- and α2,6-linked NeuAc. The α2,6-linked NeuAc was preferentially attached to the Gal(β1→4)GlcNAc(β1→2)Man (α1→3) branch. This indicates that the newly introduced ST6Gal I competes with the endogenous ST3Gal III/IV. Since the ST6Gal I from bovine colostrum has been reported to act preferentially on the Man-3 branch of diantennary glycans [71], we could confirm the specificity of the human enzyme by our *in vivo* experiments. Similarly, we showed successful α2,6-sialylation of di- and triantennary oligosaccharides of recombinant human AT III secreted by BHK-21B cells coexpressing ST6Gal I (a ratio of α2,3- to α2,6-linked NeuAc of 1:2 was determined here by integration of the NMR signals of the axial and equatorial H-3 protons of NeuAc in the total oligosaccharide mixture [16]).

Human ST6Gal I tranfers NeuAc in α2,6-linkage to GalNAc(β1→4)GlcNAc-R motifs *in vivo*

We have previously identified a BHK-21A cell clone which synthesizes large amounts of GalNAc(β1→4)GlcNAc-R motifs in addition to the common type II Gal(β1→4) GlcNAc-R structures on secretory glycoproteins [16,17]. Recombinant glycoproteins secreted from this host cell line are found to be undersialylated because the endogenous ST3Gal III/IV do not recognize terminal GalNAc(β1→4) GlcNAc residues as a substrate. This offered the possibility to investigate if the transfection of this cell line with human ST6Gal I would result in a higher degree of sialylation of recombinant EPO, since from *in vitro* experiments it has been postulated that the enzyme also recognizes LacdiNAc

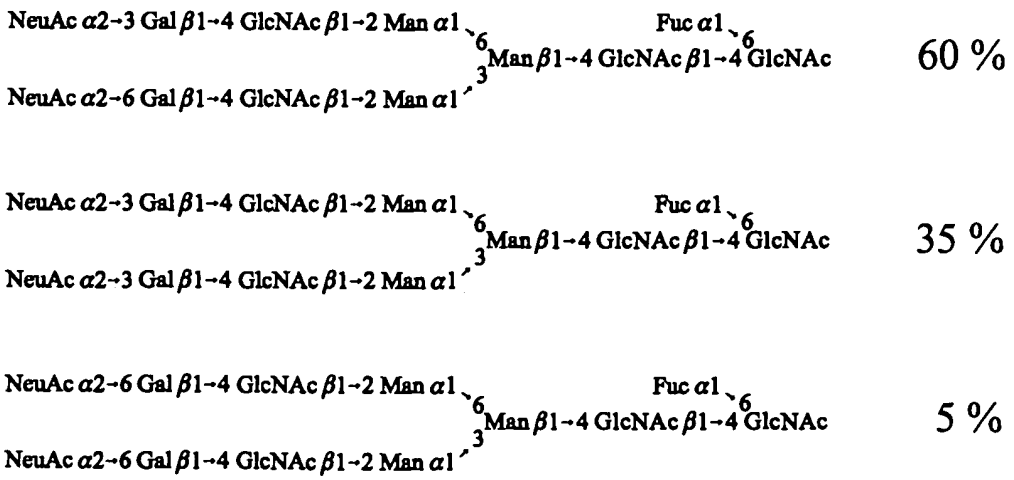

Figure 7. Disialylated *N*-linked oligosaccharide chains of recombinant β-TP expressed from ST6Gal I-transfected BHK-21B cells contain large amounts of α2,6-linked NeuAc.

motifs [72,73]. Human EPO expressed from wild-type BHK-21A cells contains large amounts of the unsialylated diantennary oligosaccharides with two terminal GalNAc(β1→4)GlcNAc motifs as well as tri- and tetrantennary chains with 1,2 or 3 GalNAc substitutions [17]. When cells are cotransfected with ST6Gal I (15-fold higher expression based on mRNA level and 5-fold higher enzyme activity with Gal(β1→4)GlcNAc-O-$(CH_2)_8COOCH_3$ as an acceptor over the endogenous α2,3-STs), we found asialo, α2,6-mono and α2,6-disialylated derivatives of the biantennary di-GalNAc oligosaccharides in a ratio of 0.1:1:0.2 based on the MALDI/TOF signals obtained from the reduced and permethylated native glycan pool of the purified EPO product (signals for $[M + K^+]$ at m/z 2360, 2721 and 3083, respectively) and methylation data [17]. This indicates that the GalNAc(β1→4)GlcNAc-R branches are efficiently recognized as substrates for ST6Gal I *in vivo*. The cell line therefore allows for the production of recombinant glycoproteins with a *human-type* NeuAc(α2→6)GalNAc(β1→4)GlcNAc-R structural motif (see Fig. 8) that is frequently found in glycoproteins secreted from human kidney tissue [74–76].

Taken together, the expression of recombinant human α2,6-sialyltransferase increases the sialylation state of glycoproteins secreted from BHK-21A and BHK-21B cells as we have shown for human β-TP, AT III and EPO [16,17]. However, the human ST6Gal I has its limitations since the enzyme has a high preference for the Man-3 branch of oligosaccharides and, due to the competition with the endogenous α2,3-STs, the final products contain mixtures of α2,3/6-sialylated oligosaccharides. Also many natural human glycoproteins have both NeuAc linkages, and at present it is unknown if therapeutic glycoproteins that are exclusively modified with α2,6-linked NeuAc would be advantageous over those with a mixture of α2,3/6-linked NeuAc.

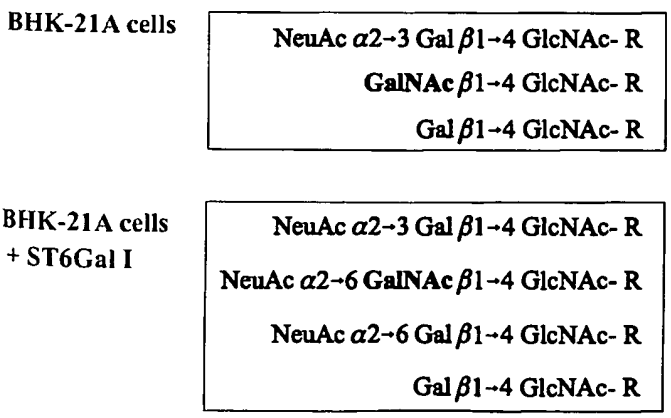

Figure 8. Terminal structural motifs in oligosaccharides of recombinant EPO from wild-type BHK-21A cells and human ST6Gal I-transfected BHK-21A cells

Future perspectives

By using recombinant DNA technology, we are now able to efficiently manipulate the glycosylation capacity of cells to be used as new stable cell factories for biotechnological processes. However, we need to understand in much more detail basic regulatory phenomena underlying the complex interaction of the intracellular enzyme machinery that is involved in the biosynthesis of glycoconjugates. All theories and current models of compartmentalization of the cellular glycosylation pathways rely on experimental data obtained by immuno-localization of the enzymes [77–79]. In our opinion, more detailed knowledge about the *in vivo* functional localization of the glycosylation machinery of cells is required and this has to be approached experimentally.

The importance of the CTS-region of glycosyltransferases not only for their *in vivo* function, but also for their *in vivo* specificity has been addressed for the human α1,3/4-fucosyltransferases in this review. In this context, it is noteworthy that polysialyltransferase (ST8Sia IV) from humans or CHO cells possesses a very short transmembrane domain of only 13 amino acid residues [80,81] which apparently does not fit to the lipid bilayer thickness model proposed for the intracellular targeting of transmembrane proteins [78,82]. Nevertheless, according to the concept of a sequential action of glycosyltransferases, ST8Sia IV should be localized within a late Golgi compartment where it can get access to its α2,3-sialylated precursor substrate, and therefore, the CTS-region of this transferase attached to the catalytic domain of, *e.g.*, FT6 should direct this enzyme into a location where it should synthesize higher amounts of sLex with coexpressed β-TP. Our results, however, obtained for this ST8Sia IV-FT6 fusion protein coexpressed together with human β-TP in BHK-21 cells, showed an oligosaccharide pattern for the reporter glycoprotein that was almost indistinguishable from that obtained from cells cotransfected with wild-type FT6 [83].

Other important considerations include:

- identification of *in vivo* functional cellular subcompartments at the molecular level of transferases and nucleotide sugar transporters
- intracellular turnover and the posttranslational modification of the enzymes
- dynamics of acceptor substrate and transferase transport within Golgi subcompartments
- role of splicing variants of glycosyltransferase/glycosidase genes

This is of general significance in all cases where recombinant glycobiology is used not only for the construction of "improved" cell factories for the production of more efficient and safer glycotherapeutics or recombinant retroviral vectors for gene therapy with oligosaccharide-based addressing signals, but also when it is intended to modify by

genetic/metabolic engineering the surface of *ex vivo* propagated human primary cells destined for *in vivo* therapy in clinics.

Acknowledgments

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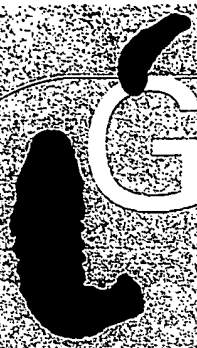

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